RAPID COMMUNICATION

Lack of Expression of Thy-1 (CD90) on Acute Myeloid Leukemia Cells With Long-Term Proliferative Ability In Vitro and In Vivo

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Acute myeloid leukaemia (AML) is thought to be maintained by a small population of leukemic progenitor cells. To define the phenotype of such cells with long-term proliferative capacity in vitro and in vivo, we have used the production of leukemic clonogenic cells (CFU) after 2 to 8 weeks in suspension culture as a measure of these cells in vitro and compared their phenotype with that of cells capable of engrafting nonobese diabetic severe combined immune deficient (NOD/SCID) mice. Leukemic blast peripheral blood cells were evaluated for expression of CD34 and Thy-1 (CD90) antigens. The majority of AML blast cells at diagnosis lacked expression of Thy-1. Most primary CFU-blast and the CFU detected at up to 8 weeks from suspension cultures were CD34$^+$/Thy-1$^-$. AML cells that were capable of engrafting NOD/SCID mice were also found to have the CD34$^+$/Thy-1$^-$ phenotype. However, significant engraftment was achieved using both CD34$^+$/Thy-1$^-$ and CD34$^-$ subfractions from one AML M5 patient. These results suggest that while heterogeneity exists between individual patients, the leukemic progenitor cells that are capable of maintaining the disease in vitro and in vivo differ from normal hematopoietic progenitor cells in their lack of expression of Thy-1.

ACUTE MYELOID LEUKEMIA (AML) is thought to arise as the result of an initial genetic change in a primitive hematopoietic progenitor cell which produces a competitive growth advantage for the resulting malignant clone.[1,2] Subsequent to transformation some evidence suggests that cells from the malignant clone undergo further differentiation, creating a hierarchy of cells that differ in their expression of cell-surface antigens and may also differ in their proliferative potential.[3-5] It has been possible to phenotypically characterize normal primitive hematopoietic cells using cell sorting combined with functional assays and to show that the expression or lack of expression of specific cell-surface antigens correlates with the maturation state of cells within the normal hematopoietic hierarchy.[6-11] In this study we have used a fluorescence-activated cell sorting (FACS) strategy to characterize expression of CD34 and Thy-1 on AML cells with long-term proliferative ability in vitro and have compared this phenotype to that of the AML cells which will engraft and proliferate in immunodeficient mice.

In the investigation of AML progenitors, most studies have focused on the ability of cells to produce small ''blast'' colonies in methylcellulose (CFU-blast), usually comprised of 10 to 100 cells per colony. The phenotype of the clonogenic cell population in AML has been shown to differ in some cases from the majority of AML cells.[3-5] This assay and other short-term in vitro assays are likely to detect progenitors with limited self-renewal and proliferative capacities.[12,13] For this reason we have used the long-term (4- to 8-week) production of CFU-blast in a serum-free recombinant growth factor–containing suspension culture as a quantitative assay for a cell that is less frequent but has a more extensive proliferative ability than CFU-blast.[14] AML progenitors capable of long-term proliferation for ≥4 weeks in this suspension culture assay have a phenotype associated with primitive normal progenitors as they are enriched in fractions selected for CD34$^+$/CD38$^-$ and CD34$^+$/CD71$^-$, whereas progenitors detected in short-term assays express higher levels of CD38 and CD71.[14]

In recent years immune deficient mice have been found to be suitable hosts for the evaluation of both normal and leukemic human hematopoietic cells in vivo.[15,16] Engraftment of human AML cells has been achieved in human growth factor–supplemented severe combined immune deficient (SCID) mice with the frequency of the SCID-leukemia initiating cells (SL-IC) estimated to range from 0.2 to 100 per 10^6 unsorted leukemic cells.[16] In some of these patients, the SL-IC activity was exclusively found in the sorted CD34^{++}/38$^-$ subfraction.[17] Although SCID mice are defective in T- and B-cell function, they display normal macrophage and natural killer (NK) cell activity.[18] The nonobese diabetic (NOD)/SCID strain is characterized by a functional deficit in NK cells, absence of circulating complement and defective antigen-presenting cells.[19] We have found engraftment of human AML cells to be more reproducible and extensive in NOD/SCID mice as compared with SCID animals.[20] Others have compared SCID and NOD/SCID mice as hosts for normal or chronic myeloid leukemia (CML) donor cells, with similar results.[21] Therefore, we have chosen detection of engraftment of AML cells in NOD/SCID mice 14 weeks postinjection as evidence for the presence, in CD34 and Thy-1 sorted subpopulations, of AML cells with long-term in vivo proliferative ability.

In the present investigation we have evaluated the expression of CD34 and Thy-1 antigens on the earliest detectable AML progenitor cells. Thy-1 (CD90) is a cell-surface glycoprotein which was originally described as a cell-surface dif-

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ferentiation marker expressed predominately in murine brain and thymus.[22-24] In humans Thy-1 is expressed on some early T and B lymphocytes, fibroblasts, and neural cells.[25,26] Although still unclear, the postulated functions of Thy-1 include cell-cell recognition events and a role in neural differentiation.[26,27] Treatment with antibody against Thy-1 has been reported to decrease the numbers of colony-forming cells (CFCs), particularly those with high proliferative potential (HPP-CFCs), suggesting that Thy-1 can mediate a negative signal which inhibits proliferation of primitive progenitor cells.[9] Cross-linking of Thy-1 on murine T lymphocytes results in their activation and cell proliferation.[28] In the hematopoietic system Thy-1 is expressed on ~25% of $CD34^+$ cells[29] and on that subfraction which is capable of reconstituting SCID mice.[30,31] We have previously shown that when $CD34^+$ cells were sorted on the basis of Thy-1 expression, the majority of cells capable of producing colonies after 5 to 8 weeks in stromal cultures (long-term culture-initiating cells) were derived from the $CD34^+/Thy\text{-}1^+$ subfraction.[29] When used in autologous bone marrow transplants, $CD34^+/Thy\text{-}1^+$ cells, isolated using high-gradient magnetic separation, are capable of engrafting and differentiating in rhesus macaque hosts.[32] Moreover, highly FACS purified $CD34^+/Thy\text{-}1^+/Lin^-$ peripheral blood cells have been shown to engraft in multiple myeloma patients with orderly maturation of erythroid, myeloid, and megakaryocyte lineages and kinetics only slightly delayed from that achieved with unpurified blood cells.[33] Together these results suggest that normal $CD34^+/Thy\text{-}1^+$ cells have stem cell properties.

To determine whether AML cells have the same pattern of expression of CD34 and Thy-1 as that described for normal hematopoietic progenitors,[29,30] we have evaluated the coexpression of CD34 and Thy-1 on AML cells with long-term proliferative ability both in vitro, using the suspension culture assay, and in vivo using sublethally irradiated NOD/SCID mice.

MATERIALS AND METHODS

Patient cells. Peripheral blood cells were obtained from patients at diagnosis of AML and in a few cases acute lymphoblastic leukaemia (ALL) after informed consent. Blood cells were Ficoll (Pharmacia Biotech, Uppsala, Sweden) separated to obtain a mononuclear cell population, then frozen in Dulbecco's modified Eagle's medium (DMEM) (StemCell Technologies Inc, Vancouver, BC, Canada) with 30% fetal calf serum (FCS) (StemCell Technologies Inc) and 10% dimethylsulfoxide (DMSO), and stored at −135°C.

AML cell phenotyping and sorting. Thawed AML cells were suspended in HFN (Hanks' medium + 2% FCS and 0.1% sodium azide) at 10^7 cells/mL. Cells were stained for 30 minutes on ice with monoclonal antibodies 8G12 (anti-CD34)[34] that had been directly coupled to the fluorochrome cyanine5-succinimidylester (Cy5) and 5E10 (anti-Thy-1)[29] coupled to phycoerythrin (PE) or OKT9 (anti-CD71)[35] labeled with fluorescein isothiocyanate (FITC) or CD38 labeled with PE (Becton Dickinson, San Jose, CA). All antibodies were used at 1 μg/mL with the exception of 8G12-Cy5, which was used at 4 μg/mL. Cells were then washed twice in HFN at 4°C, propridium iodide (PI) at 2 μg/mL was added to the cells before the second wash, and the cells were maintained on ice before phenotyping and sorting.

Cells were sorted on a dual laser FACStar^{plus} (Becton Dickinson) on the basis of fluorescence intensity after gating out nonviable cells based on PI uptake. Fractions were sorted into Iscove's Modified Dulbecco's Medium (IMDM) (StemCell Technologies Inc) with 50% FCS in microcentrifuge tubes at 4°C. Sorted fractions were washed, resuspended at known cell concentrations, and used to initiate CFU-blast and suspension culture assays or injected into NOD/SCID mice. Phenotypic analysis was performed on a FACScan (Becton Dickinson) with gating out of nonviable cells based on PI uptake.

Blast colony assays (CFU-blast). FACS-sorted peripheral blood cells, immediately following sorting, or cells removed during media changes after at least 2 weeks in suspension culture and every other week thereafter, were plated in α-methylcellulose culture medium (Methocult M3230, StemCell Technologies Inc) containing the following growth factors: 3 U/mL recombinant human (rh) erythropoietin (StemCell Technologies Inc), 20 ng/mL rh interleukin-3 (rhIL-3; Sandoz, Basel, Switzerland), 20 ng/mL rhIL-6 (Terry Fox Laboratory), 20 ng/mL rh granulocyte colony-stimulating factor (rhG-CSF; Amgen Canada Inc, Mississauga, Ontario, Canada), 20 ng/mL rh granulocyte-macrophage CSF (rhGM-CSF; Sandoz), and 50 ng/mL rhSteel Factor (Terry Fox Laboratory). After 14 days of incubation at 37°C in a 5% CO_2 humidified incubator, AML blast clusters (10 to 20 cells) or colonies (>20 cells) were counted under an inverted microscope and the numbers pooled to obtain CFU-blast counts. AML progenitors produced small dispersed colonies compared with colonies derived from normal progenitor cells. There was very little erythroid differentiation and uniform colony morphology was observed within individual patients.

Suspension culture assays. Suspension cultures were initiated with unsorted AML cells or with FACS-sorted AML peripheral blood cells at up to 10^6 cells/mL in serum-free medium (SFM) consisting of IMDM containing 10 μg/mL insulin, 200 μg/mL transferrin, 2% bovine serum albumin, 0.9% $NaHCO_3$ (StemCell Technologies Inc), and the recombinant growth factors in the concentrations described above for the CFU-blast assay. Cultures were established in 1 mL Corning multiwells (Corning CostarCorp, Cambridge, MA) or Nunclon 4 multiwells (Canadian Life Technologies, Burlington, Ontario) and maintained at 37°C in a 5% CO_2 humidified incubator. Cultures were demi-depopulated weekly by removal of half the cells plus media and replacing it with fresh media. Every second week the cells that were removed were washed twice in DMEM and cultured in α-methylcellulose to determine the CFU content of the suspension culture. Suspension cultures were maintained for 8 weeks, or until CFU could not be detected at sampling, then the entire contents of the wells were assessed for CFU content. To allow comparisons between experiments, the proportion of progenitors derived from each sorted fraction at each time point was determined by comparison to a progenitor recovery of 100% from all fractions at that time point.

Transplantation of leukemic cells in to NOD/SCID mice. NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME) were bred and maintained in sterile microisolater cages. Twenty-four hours before transplantation, mice were irradiated with 4 Gy γ irradiation from a ^{137}Cs source at a dose rate of 1.29 cGy/min. Unsorted and AML cells sorted for coexpression of CD34 and Thy-1 antigens were suspended in 0.2 mL Alpha Minimal Essential Medium (StemCell Technologies Inc) with 5% FCS and injected intravenously into the lateral tail vein of NOD/SCID mice. In the two final experiments, 10^6 irradiated normal human marrow cells, which by themselves never produced detectable $CD45^+$ cells in the mice, were coinjected with sorted subfractions. Six micrograms of human IL-3 and 10 μg human Steel factor were injected subcutaneously 3 times per week and animals were killed at 14 weeks postinjection or when they exhibited clinical signs of the disease (ruffled fur, weight loss, lethargy, limb paralysis). Five animals had to be killed in these conditions at 4 to 9 weeks postinjection. The gross anatomy of each

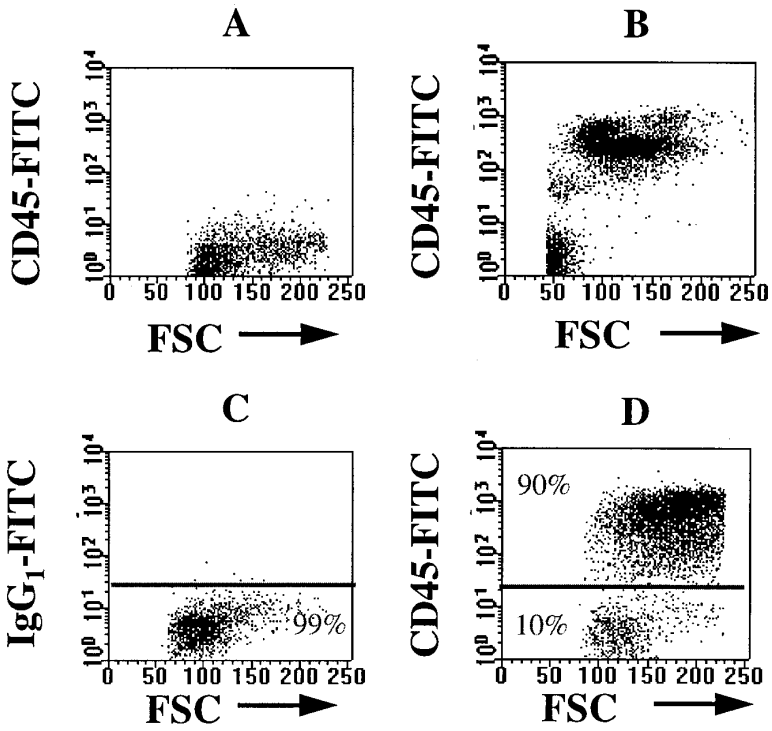

Fig 1. Analysis of human cell engraftment in NOD/SCID mice. Murine bone marrow cells (A) and human peripheral blood cells (B) were stained with CD45 as negative and positive controls for antibody specificity. Cell samples were stained with IgG_1 as an isotype control (C) and gates were set to exclude ≥99% of cells in the matched isotype control. This gate was then used to determine the proportion of positive cells in the CD45-stained murine samples (D). FSC, forward light scatter.

mouse was inspected and tissues were removed for flow cytometry, fluorescence in situ hybridization (FISH), and histologic analysis.

Flow cytometry analysis of murine tissues. Cell suspensions from blood, hind bone marrow, and spleen were incubated on ice with ammonium chloride for 20 minutes, to lyse the red blood cells, and then washed in HFN with 5% human serum. Cells from all three tissue samples were stained with a human pan leukocyte antibody, 9.4 (anti CD45),[36] directly coupled to FITC for 30 minutes on ice. Separate aliquots were stained with an irrelevant IgG_1 antibody labeled with FITC (Becton Dickinson) as an isotype control. Aliquots of normal human peripheral blood cells and bone marrow cells from a nonirradiated NOD/SCID mouse were also stained with CD45-FITC or the isotype control to serve as positive and negative controls for antibody specificity using methods as described.[37] After staining, the cells were washed in HFN containing PI (2 μg/mL) and resuspended in 300 μL HFN. Samples were analyzed using a FACScan (Becton Dickinson), nonviable cells were gated out based on PI uptake, and isotype controls were run for each tissue sample in every experiment and used to set the gates. Cells were defined as positive using gate settings which excluded ≥99% of the cells in the matched isotype control (Fig 1). For further accuracy, the percentage of positive cells in the isotype control (ie, ≤1%) was then subtracted from the percentage positive in samples stained with CD45-FITC. Whenever possible the $CD45^+$ cells derived from murine tissue were sorted and set up in short-term suspension cultures (≤3 days) or plated into methylcellulose with recombinant growth factors. The derived colonies were plucked onto slides and cells from the suspension cultures were transferred directly onto slides for subsequent cytogenetic analysis.

Cytogenetic analysis. Directly sorted cells, colonies from CFU-blast, or CFU-blast derived from subculture of long-term suspension cultures were evaluated for the leukemia-specific cytogenetic change by either standard cytogenetics or by FISH, whenever possible, in the in vitro studies. Likewise, sorted $CD45^+$ cells from tissue removed from NOD/SCID mice after a brief (≤3 days) culture and/or CFU-blast derived from directly sorted cells were evaluated for the leukemic karyotype. For analysis of CFU-blast, pools of five colonies were plucked and plated onto slides after synchronization of dividing cells in metaphase as previously described.[38] In some cases multiple five-colony pools from a plated subfraction were evaluated and the mean presented. Whole chromosome 9 painting probe directly labeled with spectrum green (BRL Life Technologies, Gaithersburg, MD) was used to detect the t(9;11) in one patient. The inversion (inv) 16 probe directly labeled with digoxigenin (Oncor, Gaithersburg, MD) was used to detect inv 16 in two other patients. The centromere specific chromosome 8 probe directly labeled to digoxigenin (Oncor) was used to identify at least one additional chromosome 8 in six other patients. In one patient the Y probe[39] was used to detect a −Y, which was uniformly associated with t(8;21) in the diagnostic cytogenetics in this patient. Standard G-banded cytogenetics[40] was used to detect +13 and del 11q23 in two other patients. The +8 probe and inv 16 probes were hybridized in Hybrisol VI (Oncor) with amplification of the signal by rabbit-anti-sheep FITC (Jackson Immuno Research Laboratories, West Grove, PA) and detection by sheep-antidigoxigenin-FITC (Boehringer Mannheim, Quebec, Canada). Counterstaining was in PI at 200 ng/mL in Vectashield (Vector Laboratories, Inc, Burlingame, CA). The chromosome 9 probe was hybridized according to the manufacturer's instructions with counterstaining as above. Whenever both metaphase and interphase cells could be scored a mean of 200 cells were counted per 5-colony pool.

RESULTS

Coexpression of CD34 and Thy-1 on circulating acute leukemia cells. Ficoll-separated blood mononuclear cells from 52 AML patients and 11 ALL patients at diagnosis or relapse were analyzed by flow cytometry for coexpression of CD34 and Thy-1, CD71, or CD38 (Table 1). A large proportion of patients in both groups had high expression of

Table 1. Proportion of Acute Leukemia Patients With Cells in CD34 Subset Fractions

% of Cells in Subfraction	$CD34^+$ (%)	$CD34^+/71^-$ (%)	$CD34^+/38^-$ (%)	$CD34^+/Thy\text{-}1^+$ (%)
AML patients				
15-100	25/52 (48)	9/48 (19)	3/50 (6)	0/52 (0)
5-15	7/52 (13)	10/48 (21)	7/50 (14)	1/52 (2)
1-5	9/52 (17)	8/48 (17)	12/50 (24)	2/52 (4)
<1	11/52 (21)	21/48 (44)	28/50 (56)	49/52 (94)
ALL patients				
15-100	8/11 (73)	5/10 (50)	0/11 (0)	2/11 (18)
5-15	1/11 (9)	1/10 (10)	1/11 (9)	0/11 (0)
1-5	2/11 (18)	3/10 (30)	9/11 (82)	3/11 (27)
<1	0 (0)	1/10 (10)	1/11 (9)	6/11 (54)

CD34 (15% to 100% of cells positive). The mean ± SEM white blood cell count of the AML patients was 88 ± 13 × 10^9/L with 65% ± 4% of circulating cells being leukemic (blasts by morphology plus monocytes in 8 cases of M5b). Most circulating cells were leukemic in all of the $CD34^+$ groups: CD34 high (15% to 100% $CD34^+$, 63% ± 6% leukemic), CD34 intermediate (5% to 15% $CD34^+$, 64% ± 10% leukemic), CD34 low (1% to 5% $CD34^+$, 52% ± 13% leukemic), and undetectable CD34 (<1% $CD34^+$, 80% ± 4% leukemic). The mean ± SEM white blood cell count of the ALL patients was 72% ± 18 × 10^9/L with 71% ± 10% being blasts.

The AML patients were French-American-British (FAB) M1 (n = 8), M2 (n = 3), M3 (n = 2), M4 (n = 20), M5 (n = 13), or other (n = 6). Of these patients, although expression of CD34 was high (mean ± SEM, 33% ± 5% $CD34^+$), almost all patients had less than 1% of cells coexpressing CD34 and Thy-1 (mean ± SEM, 0.48% ± 0.24%, $CD34^+/Thy\text{-}1^+$). This was significantly lower than the number of cells expressing CD34 and lacking expression of CD71 (10.9% ± 2.6%, $CD34^+/71^-$) or CD38 (3.0% ± 0.8%, $CD34^+/38^-$) (paired *t*-test, $P = .0002$ and .001, respectively). The proportion of $CD34^+/38^-$ cells was also significantly lower than the $CD34^+/71^-$ fraction (paired *t*-test, $P = .002$). Two ALL patients in this small group of 11 tested had high coexpression of CD34 and Thy-1.

Expression of CD34 and Thy-1 on AML cells with long-term in vitro proliferative ability. Cells from 15 patients were sorted for coexpression of CD34 and Thy-1. The clinical characteristics of these patients are shown in Table 2. Eleven of these patients had a significant number of $CD34^+$ nucleated cells (range, 29% to 92%), while in four patients only a very small proportion of cells were $CD34^+$ (0.1% to 4.2%). Nucleated cells that coexpressed CD34 and Thy-1 represented a very small fraction of cells in the blood at diagnosis in all 15 patients (mean ± SEM, 0.2% ± 0.1%, n = 15) (Fig 2). Similarly, a low fraction of CFU-blast (9% ± 9%, n = 9) and a small proportion of CFU derived from suspension culture at week 2 (0.6% ± 0.5%, n = 13), week 4 (2% ± 2%, n = 10), and weeks 6-8 (1.4% ± 1.4%, n = 7) were $CD34^+/Thy\text{-}1^+$. The higher proportion of CFU-blast from this fraction compared with other endpoints is the result of the growth of cells from a single patient with 83% of CFU-blast in this fraction. In this patient, the proportion of CFU derived from the $CD34^+/Thy\text{-}1^+$ fraction in suspension culture decreased with the length of time in culture to 19% at week 6 and 0% by week 8. Unfortunately, the few colonies produced by this $CD34^+/Thy\text{-}1^+$ fraction could not be analyzed cytogenetically because the patient had no karyotypic abnormality. Excluding this patient, the fraction of CFU-blast with this phenotype was 0.08% ± 0.04%. $CD34^+/Thy\text{-}1^-$ cells represented half of all nucleated cells at diagnosis (50% ± 9%) and the majority of CFU-blast (60% ± 14%) were $CD34^+/Thy\text{-}1^-$. Likewise, the majority of CFU detected in suspension culture at week 2 (62% ± 12%), week 4 (75% ± 11%), and weeks 6-8 (78% ± 10%) were derived from the $CD34^+/Thy\text{-}1^-$ subfraction. The remainder of all nucleated cells, CFU-blast (31% ± 14%), and CFU detected in suspension culture at week 2 (38% ± 12%), week 4 (23% ± 12%), and weeks 6-8 (21% ± 10%) were derived from the $CD34^-$ subfraction.

In two of the four patients (nos. 11 and 12) with very low numbers of $CD34^+$ cells in their blast population, the majority of CFU after 2 and 4 weeks in culture were derived from the $CD34^+/Thy\text{-}1^-$ subfraction (100% and 62%, respectively), with the proportion of cells with this phenotype increasing almost 300-fold from 0.2% of nucleated cells to 57% of CFU derived from suspension culture at weeks 6-8 in one patient. However, in the two remaining patients (nos. 9 and 10), the majority of CFU-blast (97%) and CFU derived from suspension culture at week 2 (99.6% ± 0.4%) and week 4 (100%) were $CD34^-$.

Blood mononuclear cells from 3 of these 15 patients and cells from 5 other patients were sorted on the basis of Thy-1 alone. Sorted cells were used to establish suspension cultures and the proportion of progenitor cells in each fraction was determined. The majority of nucleated cells (99.7% ± 0.1%) and CFU-blast (99.7% ± 0.2%) in all 8 patients lacked expression of Thy-1. The suspension cultures were maintained for up to 4 weeks then assessed for CFU content. As with uncultured cells, the majority of cells that were capable of producing CFU after this culture period (98% ± 1.2%) lacked expression of Thy-1.

Cytogenetic analysis of cultured cells. In experiments with nine patient samples, standard cytogenetic analysis or FISH was performed on unsorted or sorted AML cells or on plucked CFU-blast colonies, established initially or derived from suspension cultures initiated with these cell populations (Table 3). At least 70% (range, 70% to 100%) of unsorted nucleated cells analyzed had the expected chromosomal abnormality, as did $Thy\text{-}1^+$ or $CD34^+/Thy\text{-}1^+$ expressing cells which were sorted directly onto slides (69% to 99% of cells with abnormal karyotype by FISH). Similarly, primary CFU-blast and CFU from suspension culture from both unsorted or $CD34^+/Thy\text{-}1^-$ sorted cells were derived from the leukemic clone (75% ± 10%, and 74% ± 4%, respectively). In contrast, while in five experiments (four patients) most cells from primary CFU-blast derived from $Thy\text{-}1^+$ or $CD34^+/Thy\text{-}1^+$ fractions were also leukemic (83% ± 7%), most cells from CFU originating from suspension culture initiated with these fractions did not have the leukemic karyotype (28% ± 17% positive).

Table 2. Clinical Characteristics of AML Patients and Cell Growth in Baseline Cultures

Patient No.	FAB	WBC $\times 10^9$/L	% Blasts in Blood	Cytogenetic Change	CFU*/10^5	CFU in SC wk 2† (per 10^5)
1	ND	43	25	+8	1,800	1,600
2	M4	29	83	−Y, t(8;21)	3	28
3	M5	129	95	+8, del 11q23	ND	15
4	M5	47	23	del 11q23	390	860
5	M4	71	76	+8	550	75
6	M4	143	90	Normal	20	420
7	M4	410	35	+13	290	410
8	M4e	130	70	inv 16	84	0
9	M5	155	39	t(9;11)	120	1,060
10	M5	50	92	+8, +8, t(9;11)	2,170	530
11	M5	430	98	ND	14	14
12	M4	103	42	del 16q	73	10
13	M4e	65	80	inv 16	14	6
14	M4e	19	34	inv 16	770	4
15	M2	57	51	+8, t(12;22), del 20q	2,920	740

Abbreviations: ND, not determined; WBC, white blood cells.

CFU*, leukemic clusters (10-20 cells) plus colonies (>20 cells) counted after 14 days in methylcellulose culture per 10^5 cells plated.

SC wk 2†, CFU-blast in 2-week-old suspension cultures initiated with 10^5 cells. CFU numbers obtained after 2 weeks were doubled to correct for demi-depopulation at week 1.

In vivo NOD/SCID assay. Unsorted AML cells and cells sorted for coexpression of CD34 and Thy-1 from 7 of the 15 patients analyzed in vitro were also evaluated for their ability to engraft sublethally irradiated NOD/SCID mice (Table 4). Engraftment of human cells in the blood (mean percent engraftment ± SEM, 21 ± 14), bone marrow (24 ± 11), and spleen (23 ± 14) as detected by CD45 antigen expression was achieved using 1 to 5×10^6 unsorted cells from every patient. $CD34^+/Thy\text{-}1^+$ cells represented a very small fraction of the total nucleated cell population (0.14% ± 0.02%) in these experiments. The number of these cells injected ranged from 400 to 4×10^4, which represented all cells with this phenotype from $\sim 10^7$ unsorted cells. No significant engraftment was detected using these sorted cells in blood (0.1% ± 0.15%), marrow (0.3% ± 0.2%), or in spleen (0%) of recipient mice. $CD34^+/Thy\text{-}1^-$ cells represented the majority (61% ± 13%) of the total nucleated cell population in five patients ($\geq 10^6$ cells injected). Substantial engraftment (3% to 25%) was detected from one or more tissues in all recipients of the $CD34^+/Thy\text{-}1^-$ fraction with mean engraftment in blood (15% ± 9%), bone marrow (19% ± 11%), and spleen (13% ± 10%), which was not significantly different from that achieved with unsorted cells (paired *t*-test, blood $P = .7$, marrow $P = .14$, spleen $P = .24$). In two experiments $CD34^+/Thy\text{-}1^-$ cells represented less than 0.5% of the nucleated cells and only 10^4 and $3 \times$

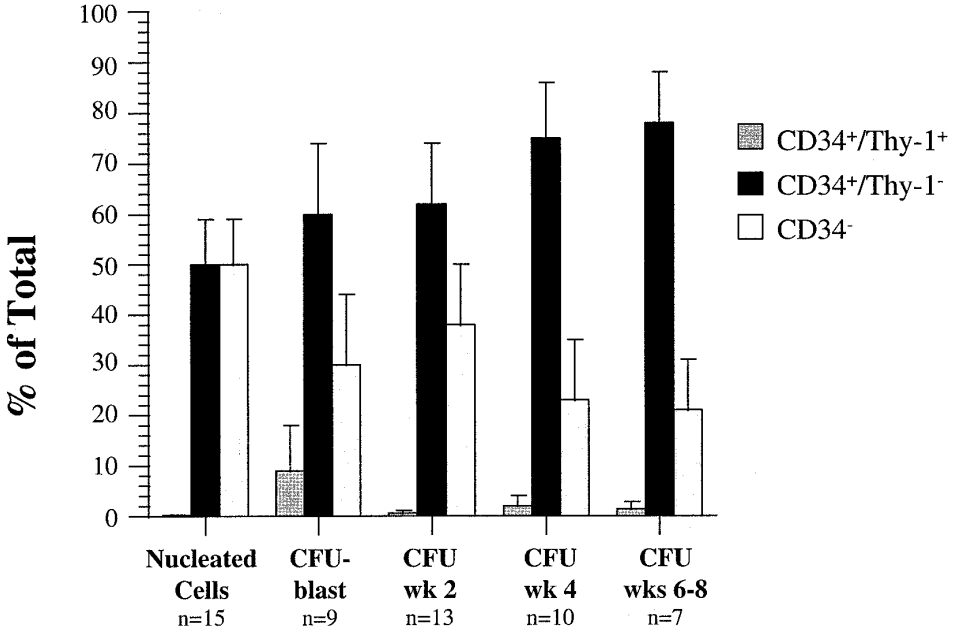

Fig 2. Proportion of AML peripheral blood cells and progenitors expressing CD34 and Thy-1 antigens from 15 patients.

Table 3. Percentage of Cells Directly Sorted or After In Vitro Assays With the Leukemic Change by FISH or Cytogenetics (no. of colonies analyzed)

Patient No.	% Positive Nucleated Cells	% Positive Cells From CFU-blast	% Positive Cells From CFU in SC
Control			
1	70	83 (20)	96 (30)
2	100	ND	ND
3	82	76 (10)	69 (20)
4	ND	ND	63 (14)
5	73	ND	76 (5)
9	ND	ND	60 (15)
10	ND	65 (5)	76 (5)
14	ND	81 (10)	ND
15	ND	81 (10)	76 (20)
Thy-1$^+$ cells			
3	89	ND	6 (20)
5	81	ND	ND
CD34$^+$/Thy-1$^+$			
1	69	91 (5)	ND
2	99	ND	ND
3	72	78 (10)	22 (10)
4	ND	ND	28 (10)
5	75	ND	29 (5)
15	ND	81 (5)	53 (5)
CD34$^+$/Thy-1$^-$			
1	ND	74 (10)	ND
4	ND	ND	70 (10)
9	ND	ND	77 (30)
10	ND	ND	68 (5)
15	ND	80 (15)	75 (20)

Abbreviations: CFU-blast, leukemic clusters (10-20 cells) plus colonies (>20 cells) plucked from methylcellulose; CFU in SC, CFU-blast derived from suspension cultures; ND, not determined.

10^4 cells with this phenotype were available for injection into mice. Nevertheless, 80% and 16% engraftment in the mouse marrow was achieved with CD34$^+$/Thy-1$^-$ cells from these two patients, respectively. CD34$^-$ cells (10^6 cells injected) from five patients, including one patient (no. 9) where CD34$^-$ cells represented 99.7% of the total nucleated cells, failed to engraft significantly in blood (0.6% ± 0.6%), bone marrow (1% ± 1%), or spleen (1% ± 1%). However, greater than 80% engraftment in all these tissues was achieved using 10^6 CD34$^-$ cells from another patient (no. 10), where this fraction represented 99.5% of total peripheral blood nucleated cells.

Cytogenetic analysis of transplanted cells. CD45$^+$ cells were sorted from tissues of NOD/SCID recipients injected with AML cells from five patients (Table 5). In all cases the expected leukemic changes were detected in cells derived from recipients of unsorted and CD34$^+$/Thy-1$^-$ fractions. More than 95% of the cells and CFU-blast derived from the blood, bone marrow, and spleen of the recipient of the CD34$^-$ subfraction from patient no. 10, who showed a high level of engraftment, were confirmed to have the leukemic karyotype. However, leukemic metaphases could not be detected in the tissues from mice injected with CD34$^-$ cells from two other patients (nos. 7 and 9) who had low levels (3% to 5%) of CD45$^+$ cells detected in some tissues.

DISCUSSION

The phenotype of the most primitive normal human hematopoietic progenitors has been difficult to determine because of the lack of available assays to measure cells with long-term multilineage marrow reconstitutive ability. As a surrogate assay for such cells we have used the long-term culture initiating cell (LTC-IC) assay, which measures a cell that produces clonogenic progenitors after more than 5 weeks in coculture with a supportive feeder layer.[41] More recently, transplantation of human cells into immunodeficient mice has been used as an in vivo assay for normal human progenitors.[15,21] Both the LTC-IC and the mouse assays predict that human cells responsible for both short- and long-term marrow reconstitution will express CD34 and Thy-1 and will lack expression of differentiation antigens.[6,29-31,42] Furthermore, transplantation of CD34$^+$ cells into both autologous

Table 4. Percentage of NOD/SCID Mouse Tissue Cells Expressing Human CD45 Antigen

Patient No.	Phenotype	No. Cells Injected	Blood*	Bone Marrow*	Spleen*
4	Unsorted	5×10^6	22	21	3
	CD34$^+$/Thy-1$^+$	6×10^3	0	0	1
	CD34$^+$/Thy-1$^-$	10^6	9	25	6
	CD34$^-$	10^6	0	0	0
5	Unsorted	5×10^6	3	0.02	0.1
	CD34$^+$/Thy-1$^+$	4×10^4	0	0	0
	CD34$^+$/Thy-1$^-$	5×10^6	20	3	0.2
	CD34$^-$	10^6	ND	ND	ND
6	Unsorted	5×10^6	2	0.3	2
	Unsorted	10^6	0.5	1	0
	CD34$^+$/Thy-1$^+$	10^4	0	0.3	0
	CD34$^+$/Thy-1$^-$	10^6	0.3	3	5
	CD34$^-$	10^6	0	0	0
7	Unsorted	10^6	3.3	49	7
	CD34$^+$/Thy-1$^+$	2×10^4	0.5	0.2	0
	CD34$^+$/Thy-1$^-$	10^6	5	8	0
	CD34$^-$	10^6	3	0	0
8	Unsorted	5×10^6	3	0.3	1
	Unsorted	10^6	1	2	0
	CD34$^+$/Thy-1$^+$	10^4	0	0	0
	CD34$^+$/Thy-1$^-$	10^6	1	0.3	3
	CD34$^-$	10^6	0	0	ND
9	Unsorted	5×10^6	89	92	85
	Unsorted	10^6	67	55	64
	CD34$^+$/Thy-1$^+$	4×10^2	1	0.1	0
	CD34$^+$/Thy-1$^-$	3×10^4	5	16	2
	CD34$^-$	10^6	0	5	5
10	Unsorted	5×10^6	96	95	94
	Unsorted	10^6	44	97	ND
	CD34$^+$/Thy-1$^+$	3×10^3	0	0	0
	CD34$^+$/Thy-1$^-$	10^4	68	80	72
	CD34$^-$	10^6	84	98	87

* The remainder of cells to 100% were CD45$^-$.

Table 5. Percentage of Cells From NOD/SCID Mice With Leukemic Change as Detected by FISH or Cytogenetics (no. of colonies analyzed)

Patient No.	Cytogenetic Change	% Positive in Blood	% Positive in Marrow	% Positive in Spleen
Control				
4	del 11q23	ND	43*	ND
7	+13	ND	21 (5)	ND
8	inv 16	86 (10)	100 (10)	ND
9	t(9;11)	60	71	60
10	+8+8, t(9;11)	80 (5)	85 (10)	ND
$CD34^+/Thy\text{-}1^-$				
4	del 11q23	67	100	ND
7	+13	22 (5)	40	ND
8	inv 16	ND	ND	83 (10)
9	t(9;11)	67	73	60
10	+8+8, t(9;11)	78 (5)	99 (6)	ND
$CD34^-$				
7	+13	0	ND	ND
9	t(9;11)	ND	0	0
10	+8+8, t(9;11)	96 (20)	97 (20)	99 (20)

* Where no number of colonies is indicated, the cells for cytogenetics were derived from $CD45^+$ cells after short-term (≤3 days) suspension culture.

and a few allogeneic hosts has resulted in prompt and sustained engraftment,[43,44] whereas transplantation of $CD34^-$ cells into baboons did not produce engraftment.[45] Autologous transplantation of further purified $CD34^+/Thy\text{-}1^+$ cells in humans[33] and in rhesus macaques[32] has also produced engraftment, suggesting that at least some stem cells are in this subfraction. $CD34^+$ cells are highly heterogeneous both in their function and in their expression of other cell-surface antigens. To compare the phenotype of normal progenitors with marrow repopulating ability with that of malignant progenitors in AML which may maintain the malignant clone in patients, we have investigated the coexpression of CD34 and Thy-1 on leukemic cells with long-term proliferative ability both in vitro and in vivo.

Our findings here indicate that very few nucleated AML cells coexpress both CD34 and Thy-1 antigens, whereas a much higher proportion are $CD34^+/CD38^-$ or $CD34^+/CD71^-$. This finding is in agreement with that of Holden et al,[42] who found that in 19 of 21 AML patients the $CD34^+$ cells lacked expression of Thy-1 antigen. When leukemic cells from 15 patients were sorted for coexpression of CD34 and Thy-1, the $CD34^+/Thy\text{-}1^+$ subfraction contributed ≤2% of cells which were capable of producing colonies after 4 to 8 weeks in suspension culture. Similar results were obtained when cells from 8 patients were stained with Thy-1 alone, suggesting that cells with long-term proliferative capacity were not present in $CD34^-/Thy\text{-}1^+$ subfraction either. Of the small number of CFU which were derived from suspension cultures initiated with $Thy\text{-}1^+$ or $CD34^+/Thy\text{-}1^+$ cells, only the minority (28% ± 8%) were found to have the genetic change. In contrast, the majority of cells that were capable of proliferation for at least 4 weeks in suspension culture were derived from the $CD34^+/Thy\text{-}1^-$ subfraction, and the majority of these were cytogenetically abnormal. Although a small number of AML progenitors may be present in the $Thy\text{-}1^+$ or $CD34^+/Thy\text{-}1^+$ subfractions, such a sorting strategy does not markedly enrich for AML progenitors while it does enrich for normal primitive progenitors.[33,46] In the present investigation the majority of nucleated cells in 4 patients were $CD34^-$. In 2 of these patients greater than 99% of CFU detected for up to 4 weeks in suspension culture were derived from the $CD34^-$ subfraction, which is consistent with other reports demonstrating the presence of CFU exclusively in the $CD34^-$ subfraction in some patients with AML.[47]

The use of immunodeficient mice as an assay for malignant human hematopoietic cells with in vivo repopulating ability has been described by several groups of investigators. Although AML cells from many patients will engraft in SCID animals with a pattern of dissemination that resembles the human disease, the variability of success with which cells from different patients proliferate in these mice and the lack of detectable engraftment in some cases suggests that residual host immunity may be reducing the sensitivity with which malignant progenitors can be detected. In the current investigation, in an attempt to overcome this potential barrier, we have used the NOD/SCID mouse which has, in addition to the SCID defect, a reduction in NK cell activity and a relative deficiency in circulating complement and antigen-presenting cells. Using these animals we have found more consistent and extensive engraftment of unsorted AML cells as compared to that achieved with SCID mice.[21] Because previous investigators had suggested that a minimum dose of 10^6 AML cells were required for engraftment, at least in SCID animals,[48] in the latter two experiments we cotransplanted 10^6 irradiated human marrow cells when the number of cells available from sorted subpopulation was small. Using these strategies, engraftment of 1 to 5×10^6 unsorted AML cells was achieved for all seven patient samples. However, there was still considerable heterogeneity between patients in the degree of engraftment achieved. Nevertheless, high levels of engraftment were achieved using 1 to 3×10^4 $CD34^+/Thy\text{-}1^-$ sorted cells from two patients in blood, bone marrow, and spleen, showing that small numbers of cells with this phenotype can engraft in NOD/SCID animals, at least in some cases. In addition, among sorted AML cells from the remaining five patients, cells that were capable of engrafting NOD/SCID mice as detected 14 weeks after injection were found exclusively in the $CD34^+/Thy\text{-}1^-$ subfraction. The leukemic nature of cells obtained from mice injected with cells from this subfraction and unsorted cells was confirmed by FISH or standard cytogenetics in five patients. In contrast, a mean of 1.3×10^4 $CD34^+/Thy\text{-}1^+$ cells derived from sorting $\sim 10^7$ AML cells, achieving an average 860-fold enrichment of cells with this phenotype, did not engraft.

In one patient the $CD34^-$ fraction also produced high engraftment and in two others a few (3% to 5%) $CD45^+$ cells were present in some tissues but they lacked the leukemic cytogenetic marker. Engraftment of $CD34^-$ cells in SCID mice in one case of AML has been reported previously.[47] These findings, together with our results, suggest that some AML patients' cells which do not express CD34 have the

ability to divide and repopulate SCID or NOD/SCID mice, unlike normal $CD34^-$ cells. Although it is possible that leukemic transformation for these patients occurred in a mature cell that no longer expresses CD34 antigen, in the present study, engraftment was also achieved with the $CD34^+$/Thy-1^- subfraction from the same patient. Thus, it is possible that transformation occurred in a $CD34^+$ cell with subsequent clonal evolution resulting in loss of CD34 expression in a subset of cells without loss of the ability to engraft.

Previous reports of engraftment of immune deficient mice with normal mobilized peripheral blood or bone marrow cells have documented that although both Thy-1^+ and Thy-1^- cells could engraft the thymus, only $CD34^+$/Thy-1^+/Lin^- cells had marrow repopulating ability.[46] As few as 100 murine Thy-1^{lo}/Lin^-/Sca^+ marrow cells will engraft and repopulate the marrow of lethally irradiated mice.[49] To our knowledge this is the first report of the characterization of Thy-1 antigen expression on primitive progenitors from patients with AML. The data we present here suggest that in most cases AML progenitor cells which have long-term proliferative capacity in vitro and the ability to repopulate sublethally irradiated NOD/SCID mice have the phenotype $CD34^+$/Thy-1^-. These cells differ from many primitive normal hematopoietic cells in that they do not express the Thy-1 antigen. Some primitive AML cells have also been found to lack CD38 and CD71 antigens, a property they share with primitive normal cells.[14,17] Whether AML cells lose Thy-1 subsequent to or upon transformation, a possibility that may have functional significance in view of its postulated role in the inhibition of proliferation of primitive cells,[9] or whether transformation occurs in a subset of normal stem cells that are $CD34^+$/$CD38^-$/$CD71^-$/Thy-1^- is unknown. The latter possibility would suggest the existence of such a population among normal stem cell populations which may have unique properties.

To gain further understanding of cells that are responsible for maintenance of the AML clone, we have focused on cells capable of long-term proliferation in vivo or in vitro. The recognition that leukemic cells may be organized in a hierarchy similar to that which exists in normal hematopoiesis has important clinical implications. To treat these diseases effectively it may be necessary to ensure that strategies successful for the elimination of the bulk of AML cells also target the cells responsible for maintaining the leukemic clone. Differences between primitive normal and malignant progenitors, such as the expression of Thy-1 reported here, may allow the development of therapeutic strategies to specifically target AML stem cells.

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