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Clonogenic Potential of Myeloid Leukaemia Cells *In vitro* is Restricted to Leukaemia Cells Expressing the CD34 Antigen

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Cells from patients with acute myeloid leukaemia (AML) or chronic myeloid leukaemia (CML) were separated into CD34-enriched and CD34-depleted subpopulations. The clonogenic capacities of these two subpopulations were then compared to each other and to the original unseparated cell population. In every study, the CD34-enriched subpopulation demonstrated a substantial increase in clonogenicity *in vitro* in comparison with the original cell population, while the reverse was the case for the CD34-depleted subpopulations. For reasons not clear at present, the enrichment for clonogenic cells far exceeded the enrichment for cells expressing the CD34 antigen. Additionally, the clonogenic potential was found to be unrelated to the level of *myc* expression in the various cell populations.
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INTRODUCTION

IN ACUTE myelogenous leukaemia (AML) myeloid maturation is arrested at or near the myeloblast level with essentially all of the leukaemia cells within a patient arrested at the same level of maturation. The leukaemia cells within an individual patient are not, however, biologically equivalent since only a subset will produce clonal growth *in vitro* [1–3] and these have a more "immature" immunophenotype than the non-clonogenic cells [4].

This report demonstrates that as with normal myeloid cells,

clonogenic AML and chronic myeloid leukaemia (CML) cells express the CD34 antigen.

MATERIALS AND METHODS

Cell acquisition

Six bone marrow (BM) aspirates and three peripheral blood (PB) specimens obtained from 5 AML and 3 CML patients were subjected to density centrifugation, (Ficol–Hyplaque = 1.077 g/cm^3; Sigma, St Louis, Missouri, U.S.A.) then the low density cells were washed and resuspended in RPMI-1640 medium (Gibco, Grand Island, New York, U.S.A.).

Cell fractionation using immunomagnetic microspheres [5]

At 10×10^6 cells/ml, the cells were incubated with an anti-CD34 monoclonal antibody (MAb) (My10) (provided by Dr Curt Civin, Johns Hopkins School of Medicine, Baltimore, Maryland, U.S.A.) (10 μg/ml 4°C, 30 min, rocker rotator). After washing, the antibody-treated cells were resuspended at 10×10^6 cells/ml and mixed with sheep antimouse IgG_1-coated magnetic microspheres (Dynabeads, Dynal, New York, U.S.A.) (0.5 microsphere per cell). The microsphere–cell mixture was vortexed, incubated (4°C, 30 min, rocker rotator) and separated using a magnet (magnetic particle concentrator MPC-1, Dynal) to hold the microsphere-bound cells to the wall of the tube. The unbound cells were poured off, constituting the CD34-depleted fraction. The microsphere cell complexes were washed and the CD34+ cells released by treatment with chymopapain [Chymodiactin, Boots Co., Lincolnshire, Illinois, U.S.A. (a gift from Dr Steve Scofield of Boots Pharmaceuticals Inc.)] (200 U/ml, 37°C, 15 min). The released cells (CD34-enriched fraction) were then poured off.

Culture of haematopoietic colony-forming cells (CFC)

Assays were performed in triplicate or quadruplicate. Unseparated or CD34-depleted cells (2×10^5) were plated in 1 ml of IMDM (Iscove's modified Dulbecco's medium, Gibco) supplemented with 25% FCS (fetal calf serum; Gibco) and 0.8% methylcellulose in 35-mm tissue culture dishes. Cultures were maintained at 37°C, 5% CO_2, 100% humidity for 14 days, CD34-enriched fractions were similarly plated but with 5×10^3/ml. Interleukin 3 (IL-3) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Genetics Institute, Cambridge, Massachusetts, U.S.A.) were used at a concentration of 100 U/ml. Phytohaemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) was prepared in our laboratory and used at a concentration of 10% v/v. Small clusters (5–20 cells), large clusters (20–40 cells) and colonies (> 40 cells) were scored at day 14.

Measurement of CD34 and c-myc expression [6,7]

Staining for CD34. Cells were washed, pelleted and resuspended in 100 μl of phosphate buffered saline (PBS) with 1% bovine serum albumin (PBS/BSA) and incubated with primary antibody (My10 or 8G12) (1 μg/10^6 cells). My10 was used for the unseparated and the CD34-depleted subpopulation, whereas 8G12 was used for the chymopapain-treated cells [8]. Cells incubated with mouse IgG_1 (1 μg/1 × 10^6 cells; Coulter Immunology, Haialeah, Florida, U.S.A.) and processed in the same way, were used as negative controls. After incubation (30 min at 4°C) the cells were washed, resuspended in 100 μl of PBS/BSA, and incubated with 3.5 μg of fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat anti-mouse Ig (TAGO Inc., Burlingame, California, U.S.A. Cat. No. 4350) at 4°C in the dark for 30 min.

Staining for c-myc [6]. Fixed cells (1% paraformaldehyde, 4°C for 30 min) were washed, permeabilised by resuspending in immunofluorescent antibody with Triton-X 100 (IFA-T; Sigma) for 5 min, pelleted and resuspended in 100 μl of IFA-T. These were incubated with a mouse MAb anti-*myc* (1 Ag/1 × 10^6 cells, 30 min, 4°C). A negative control consisted of cells treated with an anti-*myc* antibody which had been neutralised by pre-incubation with the peptide that had been used as the immunogen to prepare the antibody. The cells were then washed twice with IFA-T, resuspended in 100 μl of the same buffer and incubated with FITC-conjugated affinity-purified goat anti-mouse Ig (TAGO Inc.) (3.5 μg/1 × 10^6 cells, 30 min at 4°C in the dark). After washing, the cells were resuspended in 0.5 ml of PBS and analysed by flow cytometry.

Double staining for CD34/c-myc. Cells were incubated with My10 antibody (unseparated and CD34-depleted fraction) or 8G12 (CD34-enriched fraction), labelled with phycoerythrin(PE)-conjugated goat anti-mouse IgG1 (Fisher Biotech, Pittsburgh, Pennsylvania, U.S.A.). The cells were fixed (1% paraformaldehyde in PBS), permeabilised (0.1% Triton-X 100 in IFA buffer) and incubated with 1 μg of anti-*myc*–FITC-conjugated antibody (30 min). The cells were washed and analysed.

Flow cytometric analysis

Analyses utilised a FACScan flow cytometer (Becton Dickinson, San Jose, California, U.S.A.). Instrument settings were adjusted using CaliBRITE beads (Becton Dickinson, Cat. No. 95-0002). Forward light scatter and 90° light scatter were set to include all lymphocytes, blasts and the haematopoietic progenitor population.

Single colour analysis. Ten thousand events were collected, and the data analysed by the Consort-30 program (Becton Dickinson). Histograms created from the green signals of the negative control were used to set a marker so that more than 99% of the negative population fell to the left of the marker.

Two-colour analysis. After appropriate compensation of the FITC and PE channels using CaliBRITE beads according to the manufacturers instructions, the tube with IgG1/*myc*+ peptide was run to channelise all the cells in the left lower quadrant. Then the tube with CD34-PE/*myc*+ peptide was run to set compensation for red fluorescence. The tube with IgG1/*myc*-FITC was run to obtain compensation for green fluorescence. Finally, the tube with CD34-PE/*myc*-FITC (Fig. 1) was run. Ten thousand events were collected, stored in the LIST mode and the data analysed.

Statistical methods

The paired *t*-test procedure using log-transformed data was used to determine if the differences in total numbers of colony

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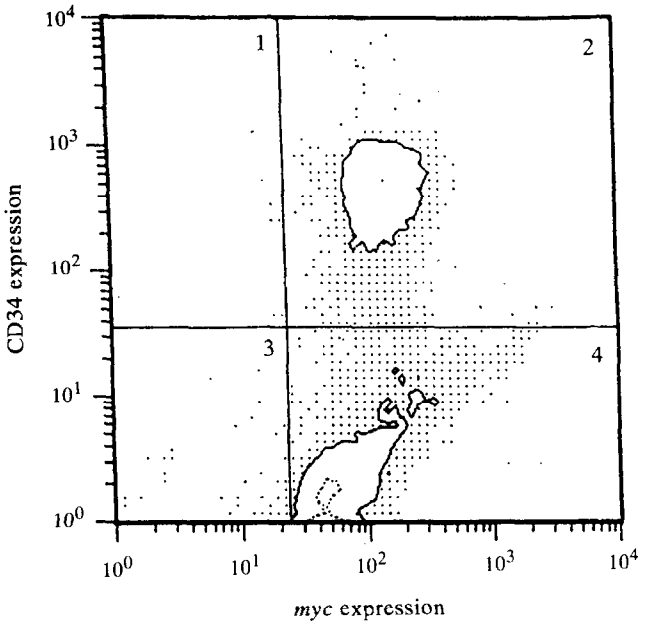

Fig. 1. Simultaneous measurement of CD34 expression and c-*myc* expression in AML cells. Note that in this patient, the leukaemia cells consist of two distinct populations, one CD34+ and one CD34−. The maximum *myc* expression is comparable in both cell populations.

forming units (CFU) in different cell populations were significantly different. The Spearman rank test was used to determine the correlation coefficients and P values.

Reagents

Monoclonal antibodies. Anti-CD34 MAb My10 was obtained from Becton Dickinson (Anti-HPCA-1 from clone My10; cat. no. 7660). 8G12 was generously provided by Dr P.M. Lansdorp (Terry Fox Laboratory, Vancouver, Canada) [8]. A monoclonal raised by immunisation of mice with a synthetic *myc* peptide was used to quantitate the *myc* protein (Microbiological Associates, Bethesda, Maryland, U.S.A.; Cat. No. P155) [9].

For double staining, an anti-*myc* antibody raised against recombinant c-*myc* protein produced in *E. coli* transformed with the pOTS-*myc* expression plasmid was used. The plasmid had been obtained from R. Watt [10]. The protein was purified as described previously [10] and used as an immunogen in mice. 10B3 gamma-globulin was prepared from BALB/c ascites and the anti-*myc* antibody purified by passing the gamma-globulin over a column composed of recombinant human c-*myc* protein coupled to Sepharose 4B. Electrophoresis showed that this procedure purified the anti-*myc* antibody to homogeneity.

Affinity-purified 10B3 anti-*myc* antibody was dialysed into coupling buffer (0.05 mol/l NaCl, pH 9.2). Ten micrograms of FITC was dissolved in dimethyl sulfoxide (5mg/ml) and reacted with 1 mg of 10B3 anti-*myc* for 2 h in the dark at room temperature. This reaction mixture was then dialysed to remove uncoupled FITC.

Buffers. IFA buffer consisted of 10 nmol/l HEPES (2.39g), 150 nmol/l NaCl (8.7g), 4% FCS (40 ml), and 0.1% sodium azide (1.0 g), all in 1 l distilled water, filtered and stored at 4°C. IFA-T was added to 0.1% Triton-X 100 IFA buffer.

RESULTS

Table 1 provides data regarding the proliferation *in vitro* of the unfractionated leukaemia cells, the CD34-enriched and the CD34-depleted subpopulations of cells. For the unfractionated cells, the mean ± S.E. number of CFU/2 × 10^5 cells for unstimulated cultures was 28.6 ± 22.7 (AML specimens 49.8 ± 6.8; CML specimens 2.7 ± 1.0). Corresponding values for PHA-LCM and IL-3-stimulated cultures were 140 ± 93 (204.7 ± 13.5 for AML; 12.0 ± 2.3 for CML) and 113 ± 56 (156.5 ± 20.0 for AML; 24.7 ± 4.0 for CML), respectively. One specimen failed to proliferate despite the presence of IL-3 or PHA-LCM.

Only one of the nine CD34-enriched subpopulations produced clonal growth in the absence of added growth factors. PHA-LCM stimulated growth in eight of the nine specimens while IL-3 stimulated growth in six of nine. The total number of CFU in PHA-LCM-stimulated CD34-enriched cell cultures was 0 to 255 times that present in the unseparated cell cultures with mean and median enrichments of 67 ± 36 and 8.4, respectively. The differences in the number of CFU produced by the CD34-enriched subpopulation and the unseparated cells was significant ($P = 0.007$).

A comparison of clonal growth by the IL-3-stimulated CD34-enriched cell populations and by the unseparated cells demonstrated a 0–23.6-fold increase in CFU number (median 5, mean 6.8 ± 2.5). This difference was also statistically significant ($P = 0.004$).

With respect to the CD34-depleted subpopulation, five specimens failed to produce growth even in the presence of exogenously added growth factors. The other four specimens produced small numbers of clones, 1/15 to 1/224 the number produced by the matched CD34-enriched subpopulations. CD34 expression was measured in three of these four specimens and a small percentage of CD34-positive cells was detected in two specimens.

CD34 and c-myc expression

The proportion of CD34+ cells and the intensity of expression were measured. CD34 expression increased from 1.5- to 100-fold (median 3.1-fold) when the unseparated and the CD34-enriched subpopulation were compared. While the intensity of CD34 expression tended to be higher in the CD34-enriched subpopulation, these differences were not significant.

There was no relationship between the percentage of CD34+ cells in a population and the number of clones produced *in vitro*.

Figure 1 provides a typical two-colour analysis of CD34 and c-*myc* expression in AML cells. While all cells contain c-*myc* protein, only a subset express CD34. The level of expression of *myc* was evaluated in six out of the nine specimens in both the unseparated and the CD34-enriched subpopulation. With respect to the unseparated specimens, 31.6–98.8% of the cells expressed c-*myc* (mean ± S.E. 78.8 ± 11.1%; median 92.5%). For the CD34-enriched subpopulation the percentage of cells expressing c-*myc* ranged from 38.3 to 89.3% with a mean (±S.E.) of 63.8 (±7.1%) and a median of 64.8%. *Myc* expression was also measured in four of the CD34-depleted subpopulations. For these cells, the percentage of c-*myc*-positive cells ranged from 32.8 to 98.8% (mean 79.4%, median 93.1%). No relationship was noted between c-*myc* expression in unseparated cells, in the CD34-enriched and CD34-depleted population and either the number of clonogenic cells in the population or the size of the CFU produced *in vitro* (data not provided).

DISCUSSION

Considering the unseparated cell population, there was no relationship between the level of CD34 expression, the level of *myc* expression and the number of clonogenic cells. On the other

Table 1. Clonal proliferation by unfractionated, CD34-enriched and CD34-depleted cell populations

Patient no.	Groups	Unfractionated				CD34+-enriched				CD34− -depleted			
		No. of CFU/ 2×10^5 cells	Size distribution*			No. of CFU/ 2×10^5 cells	Size distribution*			No. of CFU/ 2×10^5 cells	Size distribution*		
			> 40 cells	20–40 cells	5–20 cells		> 40 cells	20–40 cells	5–20 cells		> 40 cells	20–40 cells	5–20 cells
1	Control	40 ± 8	25	20	55	0	—	—	—	2 ± 1	0	0	100
	IL-3	490 ± 48	64	22	14	0	—	—	—	2 ± 1	35	23	42
	PHA-LCM	265 ± 21	74	10	16	950 ± 87	—	—	—	16 ± 6	40	0	60
							69	18	13				
2	Control	0	—	—	—	0	—	—	—	0	—	—	—
	IL-3	0	—	—	—	0	—	—	—	0	—	—	—
	GM-CSF	0	—	—	—	0	—	—	—	0	—	—	—
	PHA-LCM	0	—	—	—	250 ± 62	0	8	92	0	—	—	—
3	Control	0	—	—	—	0	—	—	—	0	—	—	—
	IL-3	28 ± 9	0	0	100	140 ± 40	0	19	81	0	—	—	—
	GM-CSF	17 ± 7	0	11	89	132 ± 40	0	0	100	0	—	—	—
	PHA-LCM	0	—	—	—	0	—	—	—	0	—	—	—
4	Control	207 ± 32	0	6	94	0	—	—	—	0	—	—	—
	IL-3	278 ± 49	0	9	91	1260 ± 202	1	26	73	65 ± 8	0	11	89
	GM-CSF	352 ± 67	0	6	94	1424 ± 46	0	25	75	72 ± 46	0	6	94
	PHA-LCM	844 ± 52	0	11	89	1455 ± 23	1	27	72	95 ± 23	0	11	89
5	Control	2 ± 1	0	0	100	0	—	—	—	0	—	—	—
	IL-3	136 ± 11	5353	26	21	1808 ± 193	62	17	21	0	—	—	—
	PHA-LCM	107 ± 4		28	19	897 ± 59	57	22	21	4 ± 2	0	0	100
6	Control	0	—	—	—	0	—	—	—	0	—	—	—
	IL-3	7 ± 3	0	43	57	165 ± 71	61	21	18	0	—	—	—
	PHA-LCM	12 ± 4	75	0	25	290 ± 48	69	12	19	0	—	—	—
7	Control	0	—	—	—	0	—	—	—	0	—	—	—
	IL-3	18 ± 6	0	6	94	134 ± 23	0	0	100	0	—	—	—
	PHA-LCM	6 ± 1	0	0	100	307 ± 6	0	0	100	0	—	—	—
8	Control	0	—	—	—	0	—	—	—	0	—	—	—
	IL-3	0	—	—	—	0	—	—	—	0	—	—	—
	GM-CSF	0	—	—	—	0	—	—	—	0	—	—	—
	PHA-LCM	20 ± 3	0	10	90	5100 ± 1103	4	33	63	0	—	—	—
9	Control	8 ± 3	0	0	100	75 ± 19	20	40	40	2±0	0	0	100
	IL-3	56 ± 6	25	14	61	435 ± 89	60	8	32	3±1	0	0	100
	PHA-LCM	10 ± 3	0	0	100	60 ± 37	100	0	0	2±2	0	0	100

*% of CFU in the different size categories. CFU, colony forming units; IL-3, interleukin 3; PHA-LCM, phytohaemagglutinin-stimulated leukocyte-conditioned medium; GM-CSF; granulocyte–macrophage colony-stimulating factor.

hand, the three AML specimens which manifested the highest level of CD34 expression (patients 1, 5 and 6) produced the largest sized colonies.

In every study the number of clonogenic cells was increased by enrichment for CD34 cells and decreased by CD34 depletion. The increase in clonogenicity in every study far exceeded the level of enrichment in the proportion of CD34-positive cells. Enrichment was not associated with an increase in clonal size.

In five studies, the CD34-depleted subpopulation failed to produce clonal growth while in three studies clonogenicity was barely detectable and probably ascribable to the persistence of CD34-positive cells. Clonogenic cells, however, were readily detectable in the CD34-depleted subpopulation of patient 4 despite the absence of detectable CD34-positive cells. Since the cloning efficiency of this specimen was significantly increased by CD34 enrichment and markedly decreased by CD34 depletion, it is possible that two clonogenic populations of cells were present, one CD34+ and another which was CD34−.

In agreement with other reports [11, 12], the data presented here suggest that, as in normal myeloid cell populations, only those leukaemia cells which express the CD34 antigen are clonogenic *in vitro*. The expression of the proto-oncogene c-*myc* in the absence of expression of the CD34 antigen, even at high levels, is not sufficient to permit cells to be clonogenic *in vitro*.

Several explanations are possible for the disproportionate enrichment in the number of clonogenic cells in the CD34-enriched subpopulation relative to the enrichment in the proportion of cells expressing the CD34 antigen: (1) the separation method employed preferentially enriches for the clonogenic cells within the CD34+ subpopulation, (2) the enrichment process removes cells which inhibit clonogenic growth, (3) above a specific concentration of CD34+ cells, the cells become more responsive to growth simulators. Which, if any, of these possibilities is correct is unknown.

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