The Presence Within Single K-562 Cells of Erythropoietic and Granulopoietic Differentiation Markers

By J. P. Marie, C. A. Izaguirre, C. I. Civin, J. Mirro, and E. A. McCulloch

The continuous cell line K-562, derived from a patient with CML in blast crisis, was examined for markers of granulopoietic (My-I) and erythropoietic (spectrin) differentiation, using specific antibodies detected by indirect immunofluorescence. Both markers were seen, and in 10%-30% of cells, both were present in the same cells. In contrast, the continuous leukemic line HL-60 and KGI contained My-I only. Controls consisted of colonies in culture containing both granulopoietic and erythropoietic cells (CFU-GEMM). In these, My-I was seen only in granulopoietic cells and spectrin in erythropoietic cells. The suggestion is advanced that genes coding for differentiation markers are expressed abnormally in K-562.

EVIDENCE IS AVAILABLE that human myelopoietic leukemia may originate in pluripotent stem cells. This conclusion has been reached by examining cells with differentiated characteristics within leukemic clones. This approach is feasible only when obviously differentiated cells are present; in their absence, capacity to differentiate along one or more pathways depends on the detection of early events in differentiation programs. Model systems have been used to approach this question. For example, Fioritoni et al. have used cytochemical and immunologic markers to demonstrate components of both erythropoietic and leukopoietic differentiation programs in murine erythroleukemic cells. The continuous cell line K-562 provides another source of material to be exploited in such studies. K-562 originated from a patient with chronic myeloblastic leukemia (CML) in the blastic phase of the disease. Morphologically K-562 cells are undifferentiated, but markers of erythropoiesis have been identified within them.

In this article we used a newly described monoclonal antibody with granulopoietic specificity to identify the presence of a component of a granulopoietic differentiation program in K-562 cells. An antispectrin antibody permitted the detection of erythropoietic differentiation events. Both erythropoietic and granulopoietic markers were found to be expressed in the same cells in approximately 10% of the K-562 population. We interpret the findings to indicate that genes coding for differentiation markers are expressed abnormally in K-562.

MATERIALS AND METHODS

Cells

K-562 was originally obtained from Lozzio and maintained in our laboratories for 6 yr. KGI and HL-60 were gifts from M. D. Minden of the Sidney Farber Cancer Center. These are leukemic cells lines and are maintained in suspension culture with 15% of fetal calf serum and a-minimal essential medium (growth medium) at 37°C in an incubator flushed continuously with 5% CO2.

Two normal bone marrow specimens were aspirated from marrow transplant donors at the time of investigation of these individuals for their suitability as donors. The protocol for obtaining specimens was approved by the Human Studies Committee of the University of Toronto.

Culture Methods

Colonies of cells of multiple myelopoietic lineages were grown using the technique of Fauser and Messner. Mononuclear cells from marrow were depleted of T-lymphocytes by removing preformed sheep erythrocyte rosettes by centrifugation through Ficoll-Hypaque (density 1.077). These T-lymphocyte-depleted mononuclear cells were plated in a viscid medium containing growth medium and 2-mercaptoethanol; stimulation was provided by erythropoietin (2 U/dish) and a medium conditioned by normal leukocytes in the presence of phytohemagglutinin (PHA-LCM). Dishes were harvested at day 6 and day 10 and tested for markers using immunofluorescence. At day 15, single mixed colonies were picked, pooled, and assayed for both My-I and spectrin-positive cells. K-562, KGI, and HL-60 cells were studied during exponential growth (2, 3, or 4 days after subculture).

Immunofluorescence

Anti-My-I is a monoclonal antibody prepared by fusion of splenic leukocytes from a mouse immunized with HL-60 and a mouse plasmacytoma cell line. The presence of the My-I antigen was detected by indirect immunofluorescence. Cells were washed three times and suspended in phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), and 0.1% sodium azide (IF medium) at a concentration of 10⁷ cells/ml. Twenty microliters were incubated with 1/10 dilution of My-I antibody at room temperature for 45 min. Cells were washed 3 times in IF medium and then incubated with a rhodamine-labeled goat IgG anti-mouse immunoglobulin (Cappel Labs, Cochraneville, Pa.) at room temperature for 30 min. After a further 3 washes in IF medium, the cells were deposited on slides by cytocentrifugation. They were fixed 5 min at —20°C with cold alcohol and washed in cold PBS overnight. The monoclonal
antibody My-1 has been shown to detect a surface marker found on normal granulopoietic cells, freshly obtained or in culture, but not on other normal cells including erythroblastic cells, erythropoietic colonies, and cultured B and T lymphocytes. A purified rabbit IgG anti-mouse spectrin was kindly provided by Dr. Eisen. Cells were washed 3 times in PBS, deposited on slides by cytocentrifugation, fixed for 10 min in cold acetone, and washed 3 times in PBS. Then, anti-spectrin (15 μl of 1/40 dilution) was placed on the slides and left for 25 min in a moist atmosphere at room temperature. The slides were washed 3 times in PBS, and fluorescein-labeled sheep IgG anti-rabbit IgG (15 μl of 1/20 dilution) was added to the slides for 25 min in a moist atmosphere. The slides were then washed overnight in PBS.

Cells were labeled with one or both antisera; coverslips were fixed to the slides with glycerol in PBS (1:1) and the slides were examined under ultraviolet epi-illumination with narrow band filters for fluorescein or rhodamine. Data were obtained from examining 200–400 cells.

For My-1, controls consisted of cells exposed to culture supernatant from parental mouse myeloma cells used in the fusion and then treated with second antibody. This control fluid is similar to the supernatant of the cloned fused cells that contains anti-My-1. Controls for anti-spectrin were cells treated either with a normal rabbit IgG and the second antibody or second antibody alone.

RESULTS

The continuous cell lines, K-562, KG1, and HL-60 were examined during exponential growth; specimens were taken and prepared for demonstration of spectrin and My-1 as described in Materials and Methods. The data are summarized in Table 1. It is apparent that approximately half of K-562 cells were positive for either My-1 or spectrin; of these between 10% and 30% contained both markers. In contrast, while HL-60 cells were usually positive for My-1, spectrin was not demonstrated within them. Only a minority of KG1 cells were My-1-positive and no spectrin-positive cells were identified.

The findings with K-562 are illustrated in Fig. 1; Fig. 1A shows cells photographed under phase microscopy. Figure 1 B and C are pictures of the same cells stained, respectively, for My-1 and spectrin. It is apparent that some cells are stained for each and an occasional cell stained for both. These doubly positive cells are indicated in the figure with arrows.

Control data are also included in Table 1. Normal bone marrow contained 38%–70% My-1-positive cells; these could readily be identified as members of the granulopoietic series. A small proportion (2%) of mature granulocytes contained large granules that stained positively with the FITC-labeled goat anti-rabbit IgG used as a developing antibody in preparations with antispectrin. These were excluded from the counts.

A second class of control consisted of colonies containing both erythropoietic and granulopoietic cells, grown in methylcellulose using the method described by Fauser and Messner. Both My-1- and spectrin-positive cells were found in such colonies; but in no case were cells identified that contained both antigens when either pooled plates or pooled mixed colonies were tested sequentially on days 6, 10, and 15 of culture.

Figure 2 illustrates the appearance of cells from mixed colonies in culture. Figure 2A taken under phase microscopy permits the identification of erythroblasts and granulopoietic cells. Figure 2 B and C show that My-1 is present in granulocytes and spectrin in erythropoietic cells.

K-562 cells have Fc receptors. To rule out binding of antibody to these receptors, unfixed cells were exposed to anti-spectrin in suspension. Less than 1% were spectrin positive in contrast to the greater than 30% positive cells found when fixed cells were exposed to antispectrin.

DISCUSSION

The data in this paper provide further evidence that the continuous cell line K-562 contains cells capable of expressing markers of more than one myelopoietic lineage. K-562 cells are morphologically undifferentiated; but glycoporin, spectrin, and synthesis of embryonic hemoglobin, markers of erythropoietic differentiation, have been reported to be present in K-562 cells. Lozzio et al. obtained equivocal results when they looked for spectrin. Our finding confirms that spectrin may be in the cell line, since it was demonstrated in cells of the clone we examined. The demonstration of My-1 provides evidence that granulopoietic markers are also expressed.

The data do not show whether or not these differentiation markers are present in clonogenic cells; to

Table 1. Analysis of MY-1 Antigen and Spectrin in Human Myeloid Cell Lines, Normal Bone Marrow, and Mixed Colonies

<table>
<thead>
<tr>
<th>Experiment</th>
<th>MY-1+ (%)</th>
<th>Spectrin+ (%)</th>
<th>MY-1+ and Spectrin+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-562</td>
<td>44.54, 19, 44.60</td>
<td>40.51, 55, 32, ND</td>
<td>10.28, 29, 9, ND</td>
</tr>
<tr>
<td>KG1</td>
<td>7.4, 3.4, 3.1</td>
<td>0.0, ND</td>
<td>—</td>
</tr>
<tr>
<td>HL-60</td>
<td>92.53, 90</td>
<td>0.0, ND</td>
<td>—</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>38.70</td>
<td>12.5*, ND</td>
<td>0</td>
</tr>
<tr>
<td>Mixed colonies</td>
<td>8.8, 46</td>
<td>66.67, 20</td>
<td>0</td>
</tr>
</tbody>
</table>

*Cells recognized morphologically as mature granulocytes but containing large cytoplasmic granules that stained with the second antibody used to demonstrate antispectrin or excluded. Such cells never were greater than 2% of the total population.
answer this question a combination of cytotoxic antibodies and colony techniques is needed, such technology is not novel and it is anticipated that the relevant information will be forthcoming.

The most striking finding reported in this article is that markers characteristic of granulopoiesis and erythropoiesis may be found together in the same cell. Fioritoni et al.6 reached a similar conclusion for Friend erythroleukemia cells on statistical grounds, although their methods did not permit direct detection of two markers in the same clone. Differentiation markers are regularly used to define hemopoietic lineages.

Normally, when components of a specific program are expressed, those of alternative lineages are absent. Our observations on colonies in culture derived from pluripotent precursors are consistent with this view of differentiation; even when such colonies were examined early, on day 6, at a time when primitive cells might be expected to be present, no examples were encountered where the same cell contained My-1 and spectrin.

In contrast, in K-562, the mechanisms that normally regulate gene expression may be defective, permitting the concurrent expression of both erythro-
DIFFERENTIATION MARKERS

poietic and granulopoietic differentiation programs. Such defective mechanisms may also explain the variation in gene expression observed in different experiments (Table 1). An abnormality of gene expression might reflect only the nature of K-562 as a highly selected line in continuous culture. However, recently we have reported\(^{18}\) that My-1 and NASD esterase are expressed in a coordinated fashion during normal granulopoiesis in culture but not when blast progenitors from patients with acute myeloblastic leukemia are examined. These latter findings were interpreted as evidence of abnormal gene expression in AML; the present data on K-562 provides further support for this concept of the nature of differentiation in myeloblastic leukemias.

K-562 contrasts with both HL-60 and KG1; these lines contain My-1-positive cells but not cells containing spectrin. The difference in rates of expression of My-1 in HL-60 and KG1 is unexplained, although consistent with variable gene expression among leukemic clones. KG1\(^{13}\) originated from a patient considered to have erythroleukemia; in this instance, therefore, the absence of evidence of erythropoietic differentiation should not be interpreted as indicating that the cell line originated in populations committed to granulopoiesis. Rather, genes coding for erythropoietic differentiation markers have either been lost or are not expressed. This view is consistent with the clinical observation that most patients diagnosed as erythroleukemia finally display the clinical features of AML,\(^{19}\) a phenotypic development observed in the patient from whose cells line KGI was derived. Potential for gene expression is now being explored in experiments in which cells are exposed to known inducers of differentiation.

Similarly, the absence of evidence of erythropoiesis in HL-60 does not exclude origin from a pluripotent precursor. Indeed, if genes are expressed abnormally in leukemia, the presence of their product in leukemic cells is not sufficient evidence to conclude confidently the nature of the cell of origin of individual clones.

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REFERENCES

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