Comparison of In Vitro Growth Characteristics of Blast Cell Progenitors (CFU-L) in Patients With Myelodysplastic Syndromes and Acute Myeloid Leukemia

By Carlo Aul, Norbert Gattermann, and Wolfgang Schneider

Current knowledge is inadequate to explain the different patterns of blast cell accumulation in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). We compared the growth patterns of blast cell progenitors (CFU-L) in 23 patients with advanced MDS and 32 patients with de novo AML. Circulating blast progenitors were identified in 74% of MDS and 81% of AML samples. Primary plating efficiencies (PE1) were similar in both disorders, despite marked differences in peripheral blast cell concentrations. By cytological and cytochemical examination, colonies from MDS patients were indistinguishable from those obtained in AML. Cell cycle status was assessed by loss of colony formation following short-term exposure to cytosine arabinoside. CFU-L suicide rates (median, range) were 40% (12% to 77%) in MDS and 60.5% (27% to 98%) in AML. Actively proliferating blast cell progenitors are thus not confined to AML, but are also present in the majority of MDS patients. An important difference between MDS and AML was found when self-renewal capacity of CFU-L was examined by means of secondary plating efficiencies (PE2). Colonies could be successfully replated in 74% of AML cases. PE2 showed marked heterogeneity (2 to 730 colonies/10⁶ mononuclear cells), with some values indicating excessive self-renewal capacity of CFU-L. In contrast, 62% of the MDS specimens failed to produce any secondary colony growth, and PE2 in the remaining cases was low (5 to 99/10⁶ MNC). We conclude that a different balance between self-renewal and determination could be responsible for a slower pace of clonal expansion in MDS, even if the proliferative activity of clonogenic cells is similar to that in AML.

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Table 1. Clinical and Hematologic Characteristics of the MDS Population

<table>
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<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis (FAB)</th>
<th>WBC (10⁸/L)</th>
<th>Blast Count (%)</th>
<th>Transformation to AML</th>
<th>Survival* (mo)</th>
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<tr>
<td>1</td>
<td>24</td>
<td>F</td>
<td>RA</td>
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<tr>
<td>2</td>
<td>61</td>
<td>F</td>
<td>RAEB</td>
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<td>4/17</td>
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<tr>
<td>3</td>
<td>64</td>
<td>F</td>
<td>RAEB</td>
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<tr>
<td>5</td>
<td>47</td>
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<td>29±†</td>
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<tr>
<td>7</td>
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<td>8</td>
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<td>RAEB/T</td>
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</tr>
<tr>
<td>9</td>
<td>73</td>
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<td>CMML</td>
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<tr>
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<tr>
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<td>52/65</td>
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<td>M</td>
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<td>65/37</td>
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<td>AML-RA</td>
<td>5.7</td>
<td>27/37</td>
<td>5</td>
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Abbreviations: FAB, French-American-British; RA, refractory anemia; RAEB, RA with excess of blasts; RAEB/T, RAEB in transformation; CMML, chronic myelomonocytic leukemia; AML-MDS, AML following MDS; PB, peripheral blood; BM, bone marrow; MNC, T-depleted mononuclear blood cells; NA, not applicable because of absent primary colony growth.

*Months from initial diagnosis of MDS.
†Treated and maintained with myelosuppressive chemotherapy.

100 mg/m² every 12 hours by infusion on days 3 to 8), daunorubicin (60 mg/m²/d intravenously on days 3 to 5), and 6-thioguanine (200 mg/m²/d orally on days 3 to 9). Of the latter patients, 14 (58%) entered complete remission (CR), as defined by Cancer and Leukemia Group B criteria, and received additional consolidation and maintenance chemotherapy according to the recommendations of the German AML Study Group. Patients who failed to achieve CR were divided into drug-resistant and inevaluable categories, following the proposals of Preider. In this classification, type I and II failures ( persistence and regrowth of leukemic cells after treatment, respectively) characterize patients with resistant disease.

Cell separation. Peripheral blood samples were used as the source of clonogenic leukemic cells in all patients. Twenty to 50 mL of blood was drawn into sterile syringes containing preservative-free heparin (10 U/mL) as anticoagulant. Mononuclear cells (MNC) of density less than 1.077 g/mL were obtained by centrifugation on a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) at 400g for 40 minutes at 20°C. Interface cells were washed three times in Hanks' buffered salt solution and adjusted to a final cell concentration of 5 x 10⁶/mL in Iscove's modified Dulbecco's medium (GIBCO, Karlsruhe, Germany), 0.9% (wt/vol) methylcellulose. For each patient, four to six Petri dishes (35 x 10 mm) were plated and incubated at 37°C in a fully humidified atmosphere supplemented with 5% CO₂. After 5 to 7 days, blast colonies containing more than 20 cells were counted under an inverted microscope (first plating efficiency, PE1). Smaller aggregates (3 to 20 cells) present in most cultures were not evaluated. In the majority of experiments, 20 to 30 primary colonies were removed from the Petri dishes with a micropipette, and analyzed for their morphology, peroxidase activity, and ability to form E rosettes.

Blast cell colony assay. Clonogenic leukemic cells were assayed using a modification of the method described by Minden et al. Briefly, 2 x 10⁵ T-depleted mononuclear cells were cultured in 1 mL IMDM containing 30% fetal calf serum (FCS) (GIBCO, Karlsruhe, Germany), 5% PHA-LCM, and 0.9% (wt/vol) methylcellulose. For each patient, four to six Petri dishes (35 x 10 mm) were plated and incubated at 37°C in a fully humidified atmosphere supplemented with 5% CO₂. After 5 to 7 days, blast colonies containing more than 20 cells were counted under an inverted microscope (first plating efficiency, PE1). Smaller aggregates (3 to 20 cells) present in most cultures were not evaluated. In the majority of experiments, 20 to 30 primary colonies were removed from the Petri dishes with a micropipette, and analyzed for their morphology, peroxidase activity, and ability to form E rosettes.

Leukocyte-conditioned medium. Phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) was produced as previously described. Using normal volunteers as leukocyte donors, several batches of PHA-LCM were prepared and tested on T-cell-depleted mononuclear blood cells (E⁻ MNC), obtained from a patient with acute myelomonocytic leukemia. Only 2 of 28 batches were found to promote the in vitro growth of clonogenic leukemic cells. These samples were mixed, stored in small aliquots at −20°C, and used as a stimulatory source throughout the whole experiment.

Ara-C suiciding. To determine the fraction of clonogenic cells in S phase of the cell cycle, 1.5 x 10⁵ T-depleted cells were incubated in 1 mL IMDM containing 1 μmol/L Ara-C for 60 minutes at 37°C, washed twice with an excess of IMDM, and plated as described above. Incubation conditions were similar to those described by Dresch et al for in vitro suicide of human bone marrow CFU-GM. An incubation time of 60 minutes was chosen.
BLAST CELL PROGENITORS IN MDS AND AML

were counted (secondary plating efficiency, because preliminary experiments had shown that suicide levels treatment.

LD Ara-C, low-dose cytosine arabinoside; MNC, T depleted mononuclear blood cells; NA, not applicable because of absent primary colony growth.

Statistical methods. Spearman rank correlations based on the ranks of actual values for paired observations were used to measure the degree of association between two variables. Group data were compared by the Wilcoxon rank sum test.

RESULTS

Primary plating efficiency of CFU-L in MDS and AML patients. Using the culture conditions described above, blast cell colonies could be grown from T depleted blood cells in 13 of 17 patients (76%) with primary MDS. In addition, colony formation was observed in four of six MDS patients first studied after progression to overt leukemia. Plating efficiency (PE1) ranged from 18 to 980/10^5 E^- MNC (median: 149/10^5). Large numbers of colonies were mainly found in patients with RAEB/T and secondary AML. As shown in Fig 1, PE1 measurements in the MDS group were significantly correlated with the concentration of blast cells in the peripheral blood (r = .63; P = .01).

In the de novo AML group, 26 of 32 samples (81%) formed colonies. PE1 varied from 15 to 932/10^5 E^- MNC (median: 110/10^5). On statistical analysis, primary plating efficiencies in MDS and AML were not significantly different. In contrast to MDS, PE1 in AML did not parallel
the peripheral blast cell concentration ($r = .15; P = .46$) (Fig 1). No correlation was found between FAB subtype and colony growth. Numbers of colonies were comparable in AML patients achieving CR after aggressive chemotherapy and in those with resistant disease. No blast cell colonies were grown from the peripheral blood of six healthy volunteers and four patients with acute lymphoblastic leukemia (2 c-ALL, 2 T-ALL), pointing to the specificity of the CFU-L assay for myeloid leukemic progenitor cells.

**Characterization of blast cell colonies.** In both disorders, CFU-L grew as compact colonies containing up to 200 cells. In 12 patients with MDS and 23 patients with de novo AML, individual colonies were removed from the methylcellulose cultures and compared for their morphology, peroxidase activity, and reactivity with sheep erythrocytes. In all cases examined, clones after 5 to 7 days of culture were mainly composed of blast cells characterized by a round or somewhat indented nucleus with a fine chromatin pattern and usually one to three prominent nucleoli. Figure 2 illustrates the morphology of a colony from a patient with CMMML. The blast cell percentage within colonies ranged from 63% to 100% and was not different between MDS and AML (median: 82 v 84%). In most cultures, the presence of some promyelocytes, myelocytes, and macrophages was noted, whereas more mature cells of the granulocytic series were never seen. In two AML patients (both FAB-M2) Auer rods were present in cultured cells, showing their leukemic origin. The level of cellular maturation was further assessed by cytochemical methods. A comparable percentage of peroxidase-positive cells was found in colonies from MDS and AML specimens (median: 21% and 15%, respectively). In both groups, contamination of culture dishes with T cell colonies was excluded by the inability of resuspended cells to form E rosettes.

**Cell cycle status.** After short-term exposure of cells to low concentrations of Ara-C (1 μmol/L), substantial loss of colony formation was observed in almost all experiments. Except for two patients with MDS (RAEB/T and CMML), CFU-L suicide always exceeded 25% (Fig 3). In some cases suicide rates were as high as 98%, indicating that all colony-forming cells were actively synthesizing DNA. In view of the wide range of values obtained, we looked to see if they were influenced by the number of primary colonies. However, a correlation between the primary plating efficiency and the percentage of CFU-L killed was neither found in the MDS group ($r = -.03; P = .93$) nor among the AML cases ($r = -.10; P = .64$). When suicide rates in MDS were compared with those in AML, a statistically significant difference could be shown. In 16 patients with MDS, the proportion of CFU-L killed showed a median of 40% (range, 12% to 77%), as compared with 60.5% (27% to 98%) in 24 patients with de novo AML ($P < .05$). Within the AML group, there was no correlation between suicide index and in vivo response of patients to aggressive chemotherapy.

**Self-renewal measurements.** The self-renewal capacity (PE2) of progenitor cells was assessed by recloning pooled primary colonies. PE2 values were available for 39 specimens. Comparing the morphology and size of primary and secondary colonies, no gross differences were detected. In five cases (three MDS and two AML), a more detailed cytological analysis was performed that confirmed the
Fig 3. Suicide rates of CFU-L after short-term exposure to Ara-C. Statistical analysis showed a significant difference between MDS and de novo AML (P < .05).

prevalence of blast cells in secondary colonies (range: 67% to 95%).

As illustrated by Fig 4, secondary plating efficiency of CFU-L was generally low in MDS. Eight of 13 samples (62%) failed to produce any secondary colonies. The remaining cases (all RAEB/T) gave rise to small numbers of secondary clones (5 to 99/10^5 MNC). Compared with the group with zero growth, these latter patients were characterized by an unfavorable clinical course, with respect to both life expectancy (median: 6 vs 13.5 months) and risk of AML development (60% vs 25%). In addition, secondary plating efficiency was assessed in three MDS patients who had already progressed to overt leukemia. Although some colony formation was observed in these cases, the plating efficiency (range: 5 to 86/10^5 MNC) did not seem to be increased in comparison with the other MDS patients. Analyzing the data on colony growth in the entire MDS group, a weak correlation was found between PE1 and PE2 values (r = .50; P = .04).

In de novo AML, primary colonies could be successfully replated in the majority of patients. Formation of secondary blast cell colonies was observed in 17 of 23 (74%) cases. In comparison with the MDS population, there was much higher patient-to-patient variation, with PE2 values ranging from 2 to 730/10^5 MNC (median: 44/10^5). In five instances the colony yield exceeded the number of primary colonies, indicating substantial self-renewal capacity of the progenitor cells. PE2 was not affected by the number of primary colonies used in the replating assay (r = .30; P = .15). Moreover, no significant correlation was found between PE2 and other variables, such as concentration of blast cells in the peripheral blood, FAB subtype, or suicide index. Figure 5 shows the PE2 values in AML patients treated with aggressive chemotherapy. A marked difference in PE2 became evident when data were arranged in two separate columns representing responders and nonresponders to chemotherapy. All patients achieving CR had secondary efficiencies of less than 60/10^5 MNC, whereas nonresponders were characterized by PE2 values ranging from 196 to 730/10^5 MNC (P < .005).

DISCUSSION

Several cell culture techniques have been developed for obtaining selective growth of leukemic cells from blood or bone marrow of patients with AML. The blast cell colony assay, first described by Buick et al., detects a subpopulation of clonogenic blood myeloblasts with high proliferative activity and self-renewal capacity that appears to be closely related to the leukemic stem cell. Using this culture method, blast colony formation can be obtained in up to
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characters of CFU-L in other hematologic malignancies. Therefore, we investigated the proliferation and self-renewal of CFU-L in 23 patients with primary MDS. Simultaneously, 32 patients with de novo AML were examined as a reference group in order to permit more meaningful interpretation of the culture data. As the growth factor requirements of blast progenitors have not as yet been defined for MDS patients, stimulation of colony growth was provided by a standard conditioned medium (PHA-LCM) that is known to contain multiple hemopoietic stimulatory activities. All cultures were performed on E-rosette depleted blood fractions to rule out interfering T-cell clone formation.

We showed that blast colony formation is not confined to AML, but also occurs in the majority of patients with advanced MDS. In the present study, circulating blast progenitors could be identified in 74% of MDS patients and 81% of AML patients. By cytological and cytochemical examination, colonies from MDS patients were indistinguishable from those obtained in AML. They mainly consisted of blast cells with no signs of terminal differentiation. Similar results have previously been published by Senn et al who, employing the same culture system, were able to detect colony-forming blast cells in 18 of 25 patients with preleukemia. They interpreted the presence of these progenitors as indicating a leukemic state in MDS. Compared with our study, they found somewhat lower plating efficiencies of CFU-L, which may be caused by differences in patient selection. Whereas the series of Senn et al mainly included RA and RARS cases, most of our patients belonged to advanced stages of MDS, often presenting with an appreciable number of blasts in the peripheral blood. Taking into account the correlation we found between peripheral blast cell concentration and colony formation, it is probably not surprising that we obtained a higher primary plating efficiency in our MDS cases. However, interpretation of primary culture data is not devoid of problems, because previous studies have shown that preculture manipulation of cell suspensions (eg, removal of T lymphocytes) and in vitro culture conditions can markedly influence colony growth.

An important characteristic of blast progenitors in AML is their substantial proliferative capacity in vitro. To examine whether this feature is shared by CFU-L from MDS patients, we performed suicide experiments in which cells were briefly exposed to Ara-C before being plated. Conditions of Ara-C incubation were similar to those described by Dresch et al for in vitro suicide of granulocyte-macrophage progenitor cells. We found a reduction in cloning efficiency of 40% in MDS patients compared with 60.5% in the AML group. Thus, we could confirm in a larger group of patients an observation made by Senn et al who reported that blast progenitors obtained from three of four patients with preleukemia were in active cell cycle. After short-term exposure to "H-thymidine, reductions in plating efficiency of 36%, 43%, and 62% were found, and this was interpreted as pointing to a similarity between the blast cell precursors identified in preleukemia and the
actively proliferating blast cell progenitors in AML. Although our comparison appears to indicate a higher proliferative capacity of CFU-L in acute myeloid leukemia, we conclude that active cell cycling is a characteristic feature of blast cell progenitors in both AML and MDS.

In contrast to these findings suggesting some similarity, our data on the self-renewal capacity of clonogenic cells show an important difference between de novo AML and MDS. Whereas in the AML group PE2 showed marked heterogeneity with some values indicating excessive self-renewal capacity of CFU-L, the secondary plating efficiency was uniformly low in MDS. This is important in view of the widely accepted notion that the clonogenic cells detected in vitro act as stem cells in vivo to maintain the leukemic cell population. According to a model proposed by McCulloch, only a minority of leukemic blast cells (usually less than 1%) show the capacity for self-renewal. The majority of the leukemic cells, on the other hand, are considered to have gone through a determination-like event that entails loss of their ability to renew themselves. The leukemic clone may thus be organized in a fashion similar to normal myelopoiesis, with a minority of blast progenitor cells generating large numbers of "differentiated" proliferatively inert leukemic cells. This model also implies that the balance between self-renewal and determination in leukemic progenitor cells is of critical importance for clonal expansion. Because self-renewing and deterministic events are considered mutually exclusive, our data suggest that the balance in MDS, as compared with AML, is in favor of determination. As determination removes cells from the renewing population, it reduces the rate of increase in clonogenic cells. A different balance between self-renewal and determination could thus be responsible for a slower pace of clonal expansion in MDS, even if the proliferative activity of clonogenic cells is similar to that in AML.

Self-renewal capacity is a rather stable biologic property of blast progenitors in each patient with AML, and several investigators have pointed out that it makes an important contribution to outcome independently of other risk factors. Patients with lower PE2 values were found to experience both higher complete remission rates and longer CR durations. We were able to confirm these results by showing that a low PE2 correlated with successful remission induction, whereas cases with extensive self-renewal were resistant to the TAD protocol. It is tempting to speculate that "resistance" in such patients is not caused by cellular drug resistance, but to the marked capacity of residual progenitor cells to self-renew and re-expand the malignant clone, despite adequate cytoreductive chemotherapy. The low PE2 measurements in our AML patients who responded well to chemotherapy were similar to the values obtained in MDS. This would seem to indicate that MDS patients may also benefit from aggressive chemotherapy. Indeed, recent studies have not confirmed the previous impression that complete remissions are much harder to achieve in MDS than in AML.

We cannot draw any conclusions as to the stability of PE2 in MDS patients during the course of their disease. In three patients with AML following MDS, some secondary colony formation was observed that did not appear greater than in the other MDS cases. However, we do not know whether these patients had any detectable secondary plating efficiency during their preleukemic phase. Only serial investigations in a sizable group of MDS patients will provide an answer to the question of whether leukemic transformation in MDS is accompanied by an increase in self-renewal capacity of clonogenic cells. Such an increase would be compatible with a multistep-tumorigenesis model, in which the myelodysplastic syndromes are initially characterized by genetic events causing mainly defective maturation, whereas further genetic damage is required to confer extensive self-renewal capacity, resulting in leukemic transformation. However, our finding of low PE2 in three patients with AML following MDS admits an alternative hypothesis. It is conceivable that progression of MDS to AML does not depend on the acquisition of extensive self-renewal capacity. Because protracted clonal expansion in MDS has already established clonal hematopoiesis, further impairment of maturation programs within the preleukemic clone may suffice to produce accumulation of blast cells. The two models may in fact complement each other. Tricot et al observed different patterns of evolution in MDS, with one group of patients showing an abrupt shift from MDS to AML and another group showing a gradual increase in bone marrow blasts with time, eventually reaching the level of clinical AML. Further study of the self-renewal capacity of CFU-L might find different mechanisms of leukemic transformation in these groups of MDS patients.

Although our finding of low PE2 in MDS helps to explain the protracted course of the disease, it must be reconciled with the fact that the preleukemic clone still comes to dominate normal hemopoiesis. Two mechanisms may be involved. First, little is known about the ability of the preleukemic clone to suppress normal hemopoiesis by production of inhibitory humoral factors. Second, even if PE2 is much lower than in AML, the probability of self-renewal of the preleukemic stem cell may no longer be closely modulated according to physiologic needs but fixed at a slightly supranormal level, perhaps by constitutive expression of an immortalizing oncogene. This should establish an abnormal growth advantage in the bone marrow and may also contribute to the in vitro detectability of CFU-L, which are not found in normal people.

Although the balance between stem cell renewal and differentiation may be determined by a small number of specific genes, the remarkable coordination of normal growth certainly involves intercellular communication, rather than relying entirely on intracellular programing. Therefore, recent studies have focused on the modulation of self-renewal through hemopoietic growth factors and cytostatic drugs, as well as on the role of the microenvironment. These experiments may open up new therapeutic possibilities by showing how self-renewal capacity, which is essential to clonal survival, may be manipulated.
REFERENCES

Comparison of in vitro growth characteristics of blast cell progenitors (CFU-L) in patients with myelodysplastic syndromes and acute myeloid leukemia

C Aul, N Gattermann and W Schneider