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Leukemic Colony-Forming Cells in Acute Myeloblastic Leukemia: Maturation Hierarchy and Growth Conditions *

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A. Introduction

Despite their primitive morphological appearance, the majority of leukemic blasts in acute myeloblastic leukemia (AML) are end-stage, nonproliferating cells. Only a small subset of AML blasts are capable of a sufficient number of divisions to form colonies in semisolid medium [1, 2]. It has been suggested that these leukemic colony-forming cells (L-CFC) may act in vivo as progenitor cells to maintain the rest of the leukemic cell population [3, 4]. L-CFC share several properties with normal myeloid progenitor cells, including self-renewal potential and high thymidine suicide index [2, 3]. As in the case of normal myeloid progenitor cells (NMPC), colony growth of L-CFC from most patients requires exogenous colony-stimulating factors (CSF) which are routinely supplied by the addition of media conditioned by activated T cells, placental tissue, or media derived from various tumor cell lines, including GCT [5], MO [6], or 5637 [7]. As NMPC proliferate in the presence of CSF, differentiate, and acquire new differentiation-associated surface antigens during this process, so L-CFC have the capacity to undergo at least limited, although abnormal, differentiation to nonproliferative cells [3, 8].

It has previously been shown that in some AML samples the majority of leukemic blasts expressed differentiation-associated antigens not present on L-CFC from the same donors [8–10], thus suggesting immaturity of L-CFC. In this study, we tested the presence of L-CFC in 62 patients with AML, using a standard agar colony assay system [11] and GCT-conditioned medium as source of CSF. Under these conditions, 21 leukemic samples did not grow in vitro, 22 formed clusters of fewer than 20 cells per aggregate, and 19 formed blast-like colonies with a buoyant density greater than 20 cells per aggregate. These 19 leukemic samples provided the basis for further studies with the goal of (a) comparing L-CFC with the majority of the leukemic cells in each sample; (b) comparing L-CFC with normal bone marrow colony-forming cells; (c) replacing GCT medium, which is known to contain a multiplicity of hematopoietic growth factor, by recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) [12] and HPLC-purified G-CSF [7] in order to identify specific L-CFC growth factors; and (d) exploring the possibility of autocrine secretion of CSF by the leukemic blast cells of some AML samples.

B. Material and Methods

I. Leukemic Cells

Peripheral blood or bone marrow aspirates were obtained at diagnosis. Leukemic cells,

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recovered by Ficoll-Diatrizoate density gradient centrifugation, were further enriched by rosetting with AET-treated sheep red blood cells to remove T cells, and by plastic adherence at 37 °C to remove monocytes. All samples tested contained more than 90% blasts. The diagnosis of AML was established by morphology, cytochemical staining, and surface antigen analysis, using a panel of monoclonal antibodies (mcAbs).

II. Colony Assay

Colonies derived from normal marrow colony-forming unit granulocyte-macrophage (CFU-GM) and L-CFC were assayed in a double-layer agar system in quadruplicate by a modification of the method of Pike and Robinson as described [13]. In this system, either highly purified NMPC (2×10^3/well) or leukemic blasts (5×10^4/well) were incorporated in the agar overlayer. CFU-GM were enumerated on days 7 and 14, L-CFC on day 10.

III. Growth Factors

GCT-conditioned medium (GCT-CM) was obtained from Gibco (Grand Island, New York). Recombinant GM-CSF was obtained from Behring (Marburg, FRG) and HPLC-purified G-CSF was a gift from Dr. K. Welte (Sloan-Kettering Cancer Center, New York, NY). In selected experiments, media conditioned by short-time (3 days)-cultured AML blasts (AML-CM) was used. Growth factors were incorporated in the agar underlayer (GCT-CM: 10% v/v; recombinant GM-CSF: 1 μg/ml; HPLC-purified G-CSF: 500 U/ml; AML-CM: 1–10% v/v).

IV. Complement Lysis

To determine the surface antigen phenotype of the CFU-GM and L-CFC, aliquots of 10^6 cells were incubated with lytic antimyeloid mcAb (1 : 250 dilutions of ascites) for 30 min at 4 °C. After two wash steps, cells were suspended in baby rabbit complement (Pel-Freez, Rogers, AR) at a dilution of 1 : 5 for 90 min at 37 °C. After two further wash steps, cells were resuspended in Iscove's modified Dulbecco's medium containing 20% fetal calf serum. Negative controls included treatment with complement alone and treatment with a lytic but nonbinding IgG monoclonal antibody (MZ4; F. Herrmann, unpublished results) and complement. Monoclonal anti-beta-2 microglobulin antibody HD46 (B. Dörken and F. Herrmann, unpublished results) was used as a positive control.

VII. Immunofluorescence Staining

Antibody reactivity with the whole leukemic cell population was determined by indirect immunofluorescence staining using a flow cytometer (Coulter Epics C) as described [13]. Negative and positive controls included the same mcAb as described above.

VIII. Monoclonal Antibodies

Besides MZ4 and HD46, a panel of five lytic antimyeloid mcAbs was used, including anti-MY9, -MZ17, -VIM2, -VIM D5, and -MY3. The reactivity of anti-MY9, -VIM2, VIM D5, and -MY3 has been described before [14]. MZ17, produced in the own laboratory, is an IgM murine mcAb which is reactive with 95% of AML samples and all permanent myeloid leukemia lines tested, including Kg1, Kg1a, HL60, HL60BII, and U937. MZ17 reacts with granulocytes, monocytes, and 3% of ALL samples but does not react with resting T cells or B cells. Preliminary biochemical data suggest that MZ17 binds to a carbohydrate moiety.

IX. Northern Blot Hybridization

In selected experiments, Northern blot hybridization of leukemic cell mRNA with complementary oligonucleotide probes corresponding to GM-CSF and G-CSF (kindly provided by Dr. D. Blohm, BASF, Ludwigshafen, FRG) was performed using standard procedures [15]. In additional experiments,

Table 1. Surface antigen phenotypes of the total AML population and of L-CFC

Patient no.	HD46		MY9		MZ17		VIM2		VIMD5		MY3		MZ4	
	Total	L-CFC[a]	Total	L-CFC	Total	L-CFC	Total	L-CFC	Total	L-CFC	Total	L-CFC	Total	L-CFC
1	+++	+++[b]	++	+++	+++	++	++	−	−	−	−	−	−	−
2	+++	+++	+	++	++	+++	+++	−	−	−	−	−	−	−
3	+++	+++	+++	++	+	+++	+	−	−	−	+	−	−	−
4	+++	+++	+++	++	+	+	++	++	++	−	−	−	−	−
5	+++	+++	++	+++	+	++	+	+++	++	−	−	−	−	−
6	++	+++	++	++	++	+++	++	+++	−	−	+	−	−	−
7	+++	+++	+	+	+	+++	++	++	++	−	−	−	−	−
8	+++	+++	++	++	++	++	+	+	−	−	−	−	−	−
9	+++	+++	+	++	+++	+++	++	+++	−	−	−	−	−	−
10	+++	+++	+	+++	+	++	+++	+++	+++	−	−	−	−	−
11	+++	+++	++	++	++	++	++	++	+	−	++	−	−	−
12	+++	+++	+	++	+	++	+	+++	++	−	−	−	−	−
13	+++	+++	+++	+++	++	++	+	+++	−	−	+	−	−	−
14	+++	+++	+	++	+++	+++	+++	+++	−	−	−	−	−	−
15	+++	+++	++	++	+	++	++	++	−	−	−	−	−	−
16	+++	+++	+	+	++	+++	++	+++	+	−	++	−	−	−
17	+++	+++	++	+++	+++	+++	++	+++	−	++	−	−	−	−
18	++	+++	+++	+++	++	+++	++	++	−	+	−	−	−	−
19	+++	+++	+++	+++	++	+++	+	++	−	++	+	++	−	−

[a] L-CFC were plated at 5×10^4/well. Results are expressed as the mean of quadruplicate cultures. Total number of L-CFC per 5×10^4 cells counted at day 10 ranged from 132 ± 17 to 148 ± 46.

[b] +, 25%–50% antigen-positive cells; ++, 50%–75% antigen-positive cells; +++, >75% antigen-positive cells.

hybridized blots were washed with boiling 0.1 × SSC/0.1% sodium dodecyl sulfate and rehybridized with a probe for the constant region of the alpha chain of the T-cell antigen receptor (kindly provided by Dr. H.D. Royer, DFCI, Boston, Massachusetts).

C. Results and Discussion

Clonogenicity of leukemic cells from 62 patients with AML was investigated in an agar culture system. In 19 of the AML samples tested, L-CFC giving rise to clonal growth into blast-like colonies was detectable. Expression of myeloid surface antigens on these L-CFC was monitored by complement lysis experiments using antimyeloid mcAb described above, and L-CFC phenotypes were compared with phenotypes of surface antigens obtained from normal bone marrow (day 7 and day 14 CFU-GM), which were assayed in an identical agar system. To investigate the relationship between L-CFC and the total leukemic cell population, immunofluorescence studies of all the leukemic populations were performed. As shown in Table 1, L-CFC formed a distinct subset of cells in AML that could be separated from the majority of the AML blasts in the same patient by immunological analysis of the surface antigen phenotype. Furthermore, surface antigen phenotypes of L-CFC showed a considerable heterogeneity among different patients. L-CFC from 3 out of 19 cases studied expressed only HD46, MY9 and MZ17; a second group of 13 cases expressed HD46, MY9, MZ17 and VIM2; in a third group of 2 cases, L-CFC were lysed with HD46, MY9, MZ17, VIM2, VIM D5, and complement, and in one case MY3 was additionally expressed.

Comparison of the phenotypes of NMPC (days 7 and 14 CFU-GM; Table 2) with L-CFC revealed that these antigens are acquired in both the normal and the malignant ontogeny in an analogous maturation-associated sequence, thus suggesting that AML can arise at multiple stages corresponding to the normal differentiation pathway. In vitro testing of L-CFC biology may be of value for the clinical evaluation of patients with AML. The identification of L-

Table 2. Surface antigen phenotypes of NMPC

Donor no.	Percent antigen-positive cells													
	Day 7–CFU-GM[a]							Day 14–CFU-GM						
	HD46	MY9	MZ17	VIM2	VIMD5	MY3	MZ4	HD46	MY9	MZ17	VIM2	VIMD5	MY3	MZ4
1	+[b]	+	+	−	−	−	−	+	+	+	−	−	−	−
	+	+	+					+	+					
	+							+						
2	+	+	+	+	−	−	−	+	+	+	−	−	−	−
	+	+	+					+	+					
	+							+						
3	+	+	+	−	−	−	−	+	+	+	−	−	−	−
	+	+	+					+	+					
	+							+						
4	+	+	+	−	−	−	−	+	+	+	−	−	−	−
	+	+	+					+	+					
	+							+						
5	+	+	+	−	−	−	−	+	+	+	−	−	−	−
	+	+	+					+	+					
	+							+						
6	+	+	+	+	+	−	−	+	+	+	−	−	−	−
	+	+	+					+	+					
	+							+						

[a] Purified NMPC were plated at 20×10^3/well. Results are expressed as the mean of quadruplicate cultures. Total number of colonies derived from day 7 CFU-GM ranged from 89 ± 4 to 181 ± 8. Number of day 14 CFM-GM ranged from 179 ± 7 to 281 ± 22.

[b] For explanation of symbols, see first footnote to Table 1.

Table 3. Presence of biologically CSF with GM-CSF properties in a medium conditioned by fresh AML cells with autonomous L-CFC growth

Source of CSF	Concentration of CSF	CFU-GM (day 14)/2×10^3 purified NMPC[a]		
		CAE+ colonies	ANAE+ colonies	LFB+ colonies
AML-CM 5	1% v/v	99	82	15
	5% v/v	100	81	15
	10% v/v	105	76	17
GCT-CM	10% v/v	101	88	17
Recombinant GM-CSF	1 μg/ml	86	75	14
HPLC-purified G-CSF	500 U/ml	150	48	1

[a] CFU-GM were enumerated at day 14. Values are expressed as colonies of quadruplicate cultures. Colonies were stained in situ for chloroacetate esterase (*CAE*; granulocytes), alpha-naphthyl acetate esterase (*ANAE*; monocytes), and Luxol fast blue (*LFB*; eosinophils).

CFC has the potential to predict effective drug combinations in cases of resistance and could also play a role in the preclinical setting of new therapies. This may be important in the evaluation of monoclonal antibodies as therapeutic in vivo agents or for in vitro purging of leukemic clonogenic cells from autologous bone marrow transplants. As in the case of normal CFU-GM, L-CFC colony growth requires the addition of exogenous growth factors, in most cases provided by CSF-containing media, although some L-CFC may be independent of exogenous CSF, probably owing to production of CSF by leukemic cells in an autocrine pathway.

We tested the L-CFC growth of 16 out of 19 AML samples in response to various sources of CSF, including GCT-CM, recombinant GM-CSF, and HPLC-purified G-CSF. In all cases, L-CFC growth occurred in the presence of GCT-CM. In each of those cases which required exogenous growth factors for L-CFC growth, recombinant GM-

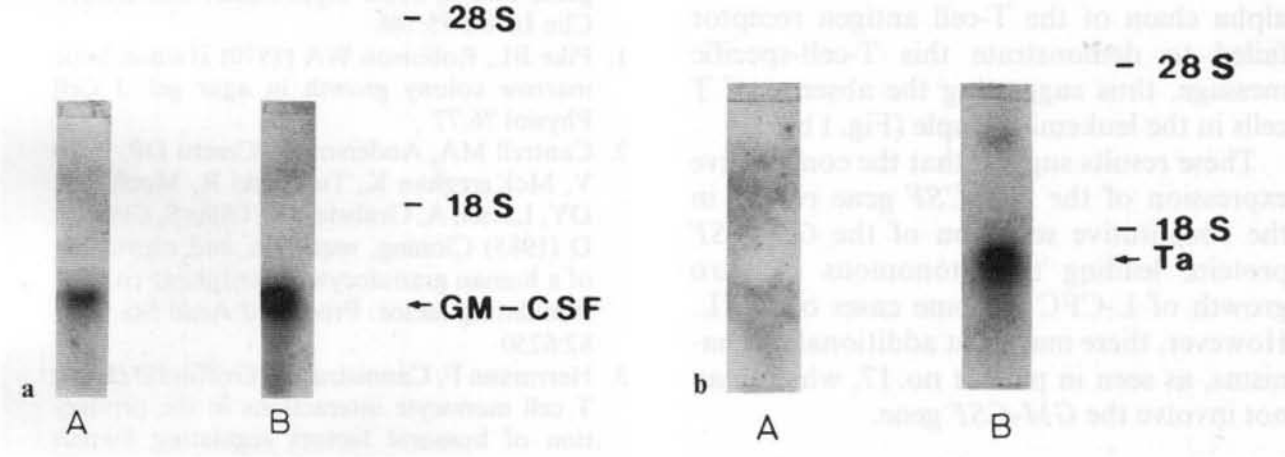

Fig. 1. a GM-CSF message of 1.0-Kb length in Northern blot analysis. Total cellular RNA (10 μg) was glyoxilated and then fractionated in a 0.8% agarose gel. RNA was transferred to a nylon membrane and hybridized to a complementary oligonucleotide (GM-CSF probe) labeled with X^{32} ATP, using T4 oligonucleotide kinase, or to a complementary Tα-DNA labeled with p^{32} dCTP, using the random primer method. *Lane A:* AML with autonomous L-CFC growth; *lane B:* phorbol myristate acetate stimulated T-lymphocytes. **b** The same blot as shown in Fig. 1 a was washed and rehybridized with a probe for the α-chain constant region of the T-cell antigen receptor, thus demonstrating the absence of this T-cell-specific message in the AML case (*lane A*) and presenting the 1.7 Kb mRNA in phorbol myristate acetate T-lymphocytes (*lane B*)

CSF but not HPLC-purified G-CSF could fully replace GCT-CM, thus suggesting that GM-CSF provides a major growth support for L-CFC in vitro (data not shown). However, in two cases, L-CFC growth occurred autonomously (patient no. 5: M2-type AML according to FAB classification; patient no. 17: M5-type AML).

It was found that medium conditioned by leukemic cells from patient no. 5 (AML-CM 5) supported normal bone marrow granulocyte, monocyte, and eosinophil colony growth (Table 3), whereas AML-CM derived from patient no. 17 (AML-CM 17) did not (data not shown). Since induction of eosinophil colony growth by AML-CM 5 suggested similarity between AML-CM 5 and GM-CSF (G-CSF and M-CSF do not induce eosinophil colony growth), we performed Northern blot hybridization of leukemic cell mRNA from this patient, using a complementary oligonucleotide for the *GM-CSF* and *G-CSF* gene respectively as probes. As shown in Fig. 1 a, Northern blot hybridization experiments with leukemic cells of patient no. 2 revealed, using the GM-CSF probe, a 1-Kb message which was indistinguishable in size from the GM-CSF message detected in phorbol myristate acetate stimulated T cells. No GM-CSF mRNA was detected in patient no. 17. G-CSF mRNA was absent in both cases (data not shown). Rehybridization of the blots with a probe for the alpha chain of the T-cell antigen receptor failed to demonstrate this T-cell-specific message, thus suggesting the absence of T cells in the leukemic sample (Fig. 1 b).

These results suggest that the constitutive expression of the *GM-CSF* gene results in the constitutive secretion of the *GM-CSF* protein, leading to autonomous in vitro growth of L-CFC in some cases of AML. However, there may exist additional mechanisms, as seen in patient no. 17, which may not involve the *GM-CSF* gene.

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