Erythroid Colony Formation in Vitro by Dimethylsulfoxide-treated Erythroleukemic Cells

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Friend leukemic cells grown in suspension culture with dimethylsulfoxide can be induced to respond to erythropoietin by increased synthesis of hemoglobin. Investigation was undertaken to determine if these erythroleukemic cells could be induced to form erythroid colonies in a plasma-clot system. This study demonstrates that culture for 24–48 hr in suspension in the presence of 2% dimethylsulfoxide affected these cells so that they then produced benzidine-staining erythroid colonies in the plasma-clot culture. The cells responded to erythropoietin in these cultures with an increase in the number of erythroid colonies.

THE PROGENY OF acute leukemic cells are characterized by a failure to complete the process of maturation. Suspension cultures of erythroleukemic cells provide a model which can be used to study the mechanisms responsible for the inability of the leukemic cells to differentiate. Under basal conditions, less than 1% of the erythroleukemic cells spontaneously differentiate along the erythroid pathway, a process which occurs independently of erythropoietin (EP).[1] When these cells are cultured in the presence of DMSO for 4 days, the percentage of differentiated cells increases to 40%–60%.[1] This is accompanied by the development of responsiveness to Ep which can stimulate a further increase in the rate of heme synthesis.[2] The nature of the erythroid differentiation by these cells and the effect of Ep on this process was investigated using the recently developed technique for the growth of erythroid colonies by normal hematopoietic cells in vitro.[3] We have found that, following treatment with DMSO, these cells are capable of erythroid colony formation. Moreover, the addition of Ep increased the numbers of such colonies.

MATERIALS AND METHODS

Friend erythroleukemic cells (clone 745A) were grown in suspension culture as previously reported.[1] Cells were harvested after growth in the absence or presence of DMSO (2% v/v) for 1–4 days. The supernatant was removed and the cells resuspended in Eagle's minimal essential medium with Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) made 2% with fetal calf serum. The following were also added: 1% nonessential amino acids (100 ×), 1% 200 m*M* L-glutamine, 1% sodium pyruvate (100 ×), 1.25% of 5% sodium bicarbonate and 1% penicillin-streptomycin mixture (Grand Island Biological Co., Grand Island, N.Y.).

The plasma-clot technique used in these studies is similar to that described by Stephenson et al.[3] One-tenth milliliter of the cell suspension containing 2×10^5 cells was added to the following mixture: 0.1 ml beef embryo extract, 0.2 ml fetal calf serum, 0.1 ml bovine serum albumin, 0.1 ml L-asparagine (0.02 mg/ml), 0.3 ml NCTC 109 (Microbiological Associates, Bethesda, Md.),

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and 0.1 ml (0.25 U) erythropoietin, step III sheep plasma Ep (Connaught Research Laboratories, Toronto, Canada).

One milliliter of this suspension was mixed with 0.1 ml of citrated bovine plasma and immediately placed in Disposo-trays (Linbro Chemical Co., New Haven, Conn.) at 0.1 ml per well in separate groups of four wells each so that each well contained about 18,000 cells. In the titration study, however, cell suspensions were prepared so that each well contained 1800, 3600, 7200, or 18,000 cells. These were placed in 35 x 10-mm petri dishes, allowed to clot, and incubated for 3-4 days at 37°C in a humidified atmosphere consisting of 5% CO_2 in room air.

After incubation the plasma clots were removed from the Disposo trays, placed onto a glass slide, and blotted with filter paper. The clots were fixed with a gluteraldehyde-buffer mixture, air dried, and placed in absolute methanol for 20 sec.

Staining for hemoglobin was carried out by placing the slides in a 1% solution of benzidine in absolute methanol for 3 min, then for 1.5 min in 3% hydrogen peroxide in 70% ethanol. The slides were then placed in distilled water for 1 min and counterstained with hematoxylin for 5 min and "blued" in running tap water.

Each clot was examined at 100 x magnification, and a group of eight cells or more was considered to be a colony. Colonies in which eight or more cells were benzidine positive (yellow to brown) were considered to be erythroid colonies.

Clots were examined after 3 and 4 days of incubation in replicate experiments. The data from day 4 cultures are shown in the tables.

RESULTS

Clots examined 2-3 hr after the initiation of culture contained only single cells. Cells from both the control and DMSO-containing cultures formed colonies in the plasma clot. By the third day of culture, colonies were extremely numerous. There was evidence of nuclear disintegration and disruption of cellular integrity in cells which had been cultured in the clot for more than 4 days.

Colonies formed by cells from control cultures consisted of undifferentiated blast cells which manifested no evidence of erythroid maturation. Figure 1 is a

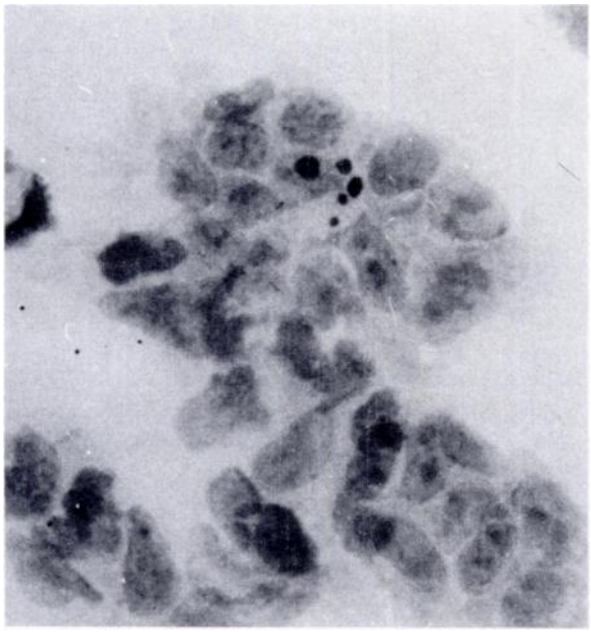

Fig. 1. Colony composed of undifferentiated blast cells.

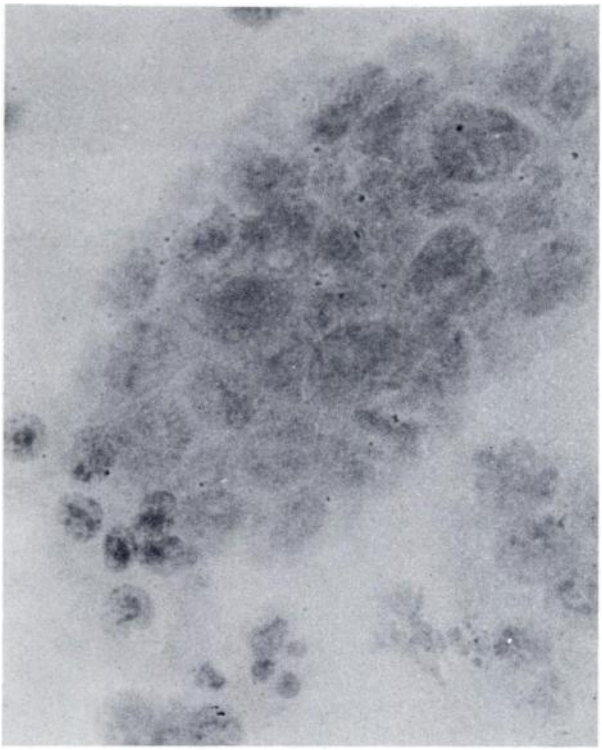

Fig. 2. Colony of erythroid and undifferentiated blast cells after incubation in DMSO.

picture of such a colony. On the other hand, erythroid colonies were present in clots which had been seeded with cells which had been grown for 1 or 2 days in suspension cultures containing DMSO. These erythroid colonies differed from those formed by normal bone marrow cells cultured in identical plasma clots. While the latter colonies consisted of a cohort of cells, all of which appeared to be at the same level of erythroid maturation, colonies derived from leukemic cells consisted of cells of varying degrees of erythroid maturation. Some cells in the leukemic colonies were blastic in appearance, while others manifested varying degrees of erythroid maturation as indicated by the development of strong benzidine positivity as well as increased pyknosis of the nucleus (Fig. 2). Colonies counted as nonerythroid often had several erythroid cells present but not enough to be classified as erythroid colonies according to the criteria set up in Materials and Methods. Cells which had been grown in suspension cultures in the presence of DMSO for 3 or 4 days produced only undifferentiated colonies in the clot. These colonies did not contain any benzidine-staining cells.

Responsiveness to Ep was detected in cells which had been cultured in the presence of DMSO for 1 or 2 days. The presence of Ep in the clot resulted in a significant increase in the number of erythroid colonies (Tables 1 and 2). Plasma clots which had been seeded with control cells or cells which had been grown in suspension culture in the presence of DMSO for 3–4 days produced only undifferentiated colonies despite the presence of Ep. The total numbers of colonies (erythroid and nonerythroid) formed in plasma-clot cultures in the

Table 1. Effect of Erythropoietin (Ep) on the Numbers of Erythroid Colonies Formed in Plasma-clot Cultures of DMSO-treated and Untreated Friend Leukemic Cells (FLC) in Vitro*

Days and Treatment in Suspension Culture		No. of Erythroid Colonies/Clot†					
		No Ep			+ Ep		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
1	None	0	0	0	0	0	0
	DMSO	9.5 ± 1.6	12 ± 2.0	7.8 ± 1.5	39.5 ± 15.06	31 ± 4.2	19.5 ± 2.4
2	None	0	0	0	0	0	0
	DMSO	16 ± 4.08	8 ± 1.5	192 ± 6.5	44.5 ± 10.28	39 ± 4.1	389 ± 5.5

*FLC were grown in suspension cultures in presence or absence of DMSO for 1–4 days; cells were harvested, washed free of DMSO and media, and cultured in plasma clot in presence or absence of Ep for 4 days. No DMSO was added to the clot culture.

†Each value represents the mean ± 1 SE of four separate clots after 4 days of culture.

absence of Ep were not significantly different from those produced in the presence of Ep (Table 2). However, cells previously treated with DMSO for 4 days produced significantly less colonies when compared to control cells. In general, addition of Ep to DMSO-treated cells did not significantly alter the total number of colonies formed in the plasma clot. On the other hand, addition of Ep to DMSO-treated cells resulted in the formation of greater numbers of erythroid colonies, so that erythroid colonies formed a significantly greater percentage of the total number of colonies formed in these plasma-clot cultures (Table 2).

Table 2. Effect of DMSO Treatment of Friend Leukemic Cells (FLC) on the Colony-forming Efficiency of FLC and the Effect of Ep on the Formation of Erythroid Colonies by These Cells in Plasma-clot Culture in Vitro*

Days and Treatment in Suspension Culture		Starting Cell Concentration/Clot	No. of Colonies/Clot†			
			No Ep		+ Ep	
			Total	Erythroid	Total	Erythroid
2	None	1800	158 ± 12	0	173 ± 11	0
		7200	474 ± 18	0	430 ± 10	0
		18,000	598 ± 22	—	617 ± 19	0
	DMSO	1800	62 ± 2	13 ± 2	69 ± 1	23 ± 0.5
		3600	115 ± 0.5	38 ± 5	123 ± 1	53 ± 2
		7200	N.D.‡	N.D.	201 ± 18	72 ± 10
		18,000	660 ± 15	192 ± 6	622 ± 32	389 ± 6
4	None	1800	141 ± 9	0	134 ± 8	0
		7200	304 ± 20	0	272 ± 22	0
		18,000	441 ± 21	0	420 ± 30	0
	DMSO	1800	6 ± 1	0	4 ± 0.3	0
		3600	10 ± 1	0	8 ± 0.9	0
		7200	N.D.	0	21 ± 2	0
		18,000	132 ± 8	0	133 ± 13	0

*FLC were grown in suspension cultures in presence or absence of DMSO for 2 or 4 days; cells were harvested, washed free of DMSO and media, and cultured in plasma clot in presence or absence of Ep for 4 days. No DMSO was added to the clot culture.

†Each value represents the mean + 1 SE of four separate clots.

‡Not determined.

DISCUSSION

The studies reported here demonstrate that Friend leukemic cells can form colonies in plasma clots. The type of colony depended upon the cell inoculum. Cells which had been cultured in DMSO for 3 or 4 days and cells from control cultures produced colonies composed of only undifferentiated cells. However, cells which had been cultured for 1–2 days in the presence of DMSO formed erythroid as well as nonerythroid colonies.

The erythroid colonies differed from those produced by normal bone marrow cells. The former colonies consisted of cells of varying degrees of erythroid maturation, while the latter were of a uniform level of maturation. This observation has at least two possible explanations: (1) The leukemic cell progenitor of the erythroid colony may have a greater capacity for replication than does the colony-forming cell present in normal bone marrow. Thus, the former cell could produce several distinct generations of progeny, while the latter cell produces only one wave of progeny which differentiates as a cohort. (2) The progeny of the erythroleukemia cell may differ from each other in the extent of their ability to differentiate, while those of the normal colony-forming cell do not. This possibility is particularly attractive since benzidine-positive cells were often present in colonies which were classified as nonerythroid according to the criteria stated in the Materials and Methods section.

We have previously reported[2] that Ep did not appear to increase the proportion of differentiating cells in a suspension culture but rather appeared to stimulate the synthesis of heme in those cells which had already begun to differentiate. On the other hand, in the studies reported here, the presence of Ep in the plasma clot resulted in an increased number of erythroid colonies. These seemingly contradictory findings may be the result of differences in the culture systems employed. Cell replication did not occur in the suspension-culture system used to study synthesis of heme, while in the plasma clot replication was prodigious. Thus, Ep may be capable of exerting a variety of effects on these cells, but the conditions of culture may determine which predominate.

The morphologic[1] and biochemical[4-6] differentiation induced by DMSO is accompanied by the acquisition of traits which are characteristic of erythroid progenitor cells. In this regard, the colony-forming ability of these cells declines significantly following DMSO-induced differentiation. This was apparent from the significantly decreased numbers of colonies formed as the time of exposure to DMSO in the primary culture was increased to 4 days (Table 2). Similar results were obtained when the colony-forming ability of these cells was examined in the soft agar cultures in the presence or absence of colony-stimulating factor.[7] It appears possible that the ability to form colonies in the plasma clot or soft agar is a property which is characteristic in these cells of a less differentiated state than is the ability to respond to Ep. The possibility that the decline in the colony-forming ability is due to toxicity of DMSO can be ruled out since (1) no DMSO was present in the clot cultures, and (2) the FLC continue to proliferate in primary cultures in the presence of 2% DMSO with no apparent toxic effect.[1] Hence the decline in colony-forming ability and the acquisition of responsiveness to Ep may reflect the progression of the cells from a relatively

undifferentiated state to a more differentiated state. Similarly, after 72 hr of culture, the differentiating cells may be too mature to act as erythroid colony progenitor cells.

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