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Cell Biology

Characterization of an erythroid precursor cell of high proliferative capacity in normal human peripheral blood

(erythropoiesis/blood stem cells/lymphocytes)

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ABSTRACT **We have found that the peripheral blood of normal humans contains a significant number of committed erythroid stem cells of high proliferative capacity. These erythroid stem cells closely resemble the murine erythroid burst-forming unit (BFU-E) with respect to proliferative capacity, colony morphology, and erythropoietin requirement. BFU-E were isolated from the peripheral blood of normal individuals by Ficoll–Hypaque density gradient centrifugation and cultured *in vitro* using the plasma culture technique. Macroscopic erythroid colonies of between 100 and 1000 cells were observed after 10–14 days of culture in the presence of either sheep or human erythropoietin at 0.5–4 units/ml. Individual colonies contained between 3 and 20 subcolonies and reached a maximum mean size of approximately 500 cells. Colony number was linearly related to the cell input, suggesting that a single cellular entity was precursor to each colony. The frequency of cells capable of giving rise to an erythroid colony was at least 100 per ml of blood in a number of individuals tested. The ability to assay significant numbers of erythroid precursor cells of high proliferative capacity from normal peripheral blood should facilitate the study of both normal erythropoiesis and of disease states affecting erythropoiesis in which marrow samples are not available on a routine basis.**

The analysis of erythropoiesis in the mouse has been advanced significantly by the development and application of techniques for cloning committed erythroid precursor cells *in vitro* (1–5). Recently, these techniques have been extended for use with human erythroid precursor cells (6, 7). In the mouse, Heath *et al.* clearly demonstrate the existence of two distinct cell populations in the marrow, BFU-E (burst-forming unit that responds to erythropoietin) and CFU-E (colony-forming unit that responds to erythropoietin) (8). These populations can be distinguished by a number of characteristics, including cell volume, responsiveness to erythropoietin, and proliferative capacity. Axelrad *et al.* (4, 8) have proposed that BFU-E, a cell which differentiates in response to a high dose of erythropoietin and has a relatively high proliferative capacity (colony size 64–1000 cells), is the precursor of CFU-E, a cell of lower proliferative capacity (colony size 8–64 cells), although direct experimental evidence to support this view is not currently available.

Previous analysis of human erythroid precursor cells has focused primarily on cells obtained from bone marrow aspirates. The extent of proliferation of cells obtained from human bone marrow has generally been less than that observed for mouse BFU-E-derived colonies. In the only previous report on erythroid precursor cells in human peripheral blood a very limited proliferative capacity was also observed (9). In the present study we demonstrate the presence in human peripheral blood of a cell with a proliferative capacity equivalent to that of murine BFU-E. The morphological appearance of the erythroid colonies derived from human peripheral blood cells bears a striking resemblance to that of mouse BFU-E colonies. The demonstration of significant numbers or proliferative capacity of early erythroid precursors in human peripheral blood could have important implications both for the understanding of normal erythropoiesis and for the analysis of disease states affecting erythrocyte formation.

Abbreviations: BFU-E, burst-forming unit that responds to erythropoietin; CFU-E, colony-forming unit that responds to erythropoietin.

MATERIALS AND METHODS

Samples (50 ml) of normal human peripheral blood were drawn and mixed with either 0.4% sodium citrate or preservative-free acid–citrate–dextrose. Mononuclear cells were separated from erythrocytes using a modification of the Ficoll–Hypaque density centrifugation technique (10). Briefly, 25 ml of undiluted blood was layered onto 25 ml of Ficoll–Hypaque (Pharmacia Ltd., Piscataway, N.J.) and centrifuged at 960 × *g* for 20 min at 20°. The mononuclear cell layer was collected, washed once in ice-cold alpha minimum essential medium containing 0.4% sodium citrate, and resuspended in alpha minimum essential medium without citrate at the desired cell concentration. The mononuclear cell fraction contained a high proportion of both monocytes and lymphocytes and was routinely observed to contain more than 50% nucleated cells after a single Ficoll–Hypaque density centrifugation. Nucleated cell concentration was determined by hemocytometer count of cells suspended in 10% acetic acid.

Further operations were carried out at 4°. Mononuclear cells were plated in a final volume of 0.1 ml in plastic microtiter wells (MRC no. 96 Linbro Chemical Co., New Haven, Conn.) using the plasma culture technique (2, 8). A modification of the technique that yielded substantially higher plating efficiencies involved replacing beef embryo extract with a final concentration of 1.0 units/ml of semi-purified bovine thrombin (Grade 1, Sigma Chemical Co., St. Louis, Mo.). Either human urinary erythropoietin (lot H-11-TaLSL, U.S. National Institutes of Health) or erythropoietin from anemic sheep plasma (Step III, Connaught Laboratories, Willowdale, Ontario, Canada) was added at the desired concentrations, 0.5–4 units/ml, at zero time. Cultures were incubated in an atmosphere of high humidity and 95% air/5% CO_2 at 37° for various periods. Squash preparations were prepared as previously described (2). Cytocentrifuge preparations were obtained by releasing cells from clots by treatment of 0.1 ml of clot with 0.3 ml of Pronase at 1 mg/ml (Grade B, Calbiochem, La Jolla, Calif.) dissolved in phosphate-buffered physiologic salt solution for 2 min and pelleting the released cells onto a slide at 500 rpm for 5 min in a Shandon cytocentrifuge. ^{59}Fe radioautography was performed essentially as described (1). Cultures were labeled from days 7–12 by the addition of 1 μCi of human-transferrin-bound ^{59}Fe in a volume of 10 μl.

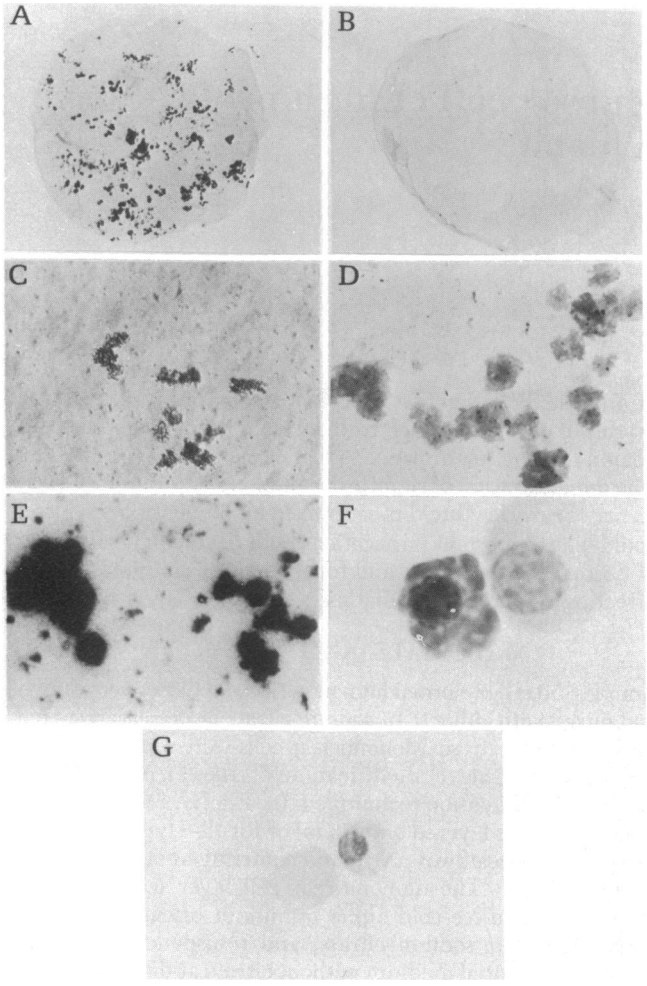

FIG. 1. (A) Squash preparation of a plasma culture seeded at 2 × 10^5 nucleated cells per culture, erythropoietin at 4 units/ml. The preparation was stained with benzidine–peroxide reagent and counterstained with hematoxylin. After 14 days of incubation this culture contained 29 BFU-E-derived colonies. Original diameter of the squashed clot was 7 mm. (B) Parallel culture to that in *A*, without erythropoietin. No BFU-E-derived colonies are visible. (C) Phase-contrast photomicrograph (32×) of a typical grouping of subcolonies within a BFU-E-derived colony in the plasma clot. (D) A mature BFU-E-derived colony containing 13 subcolonies. (Benzidine–hematoxylin stain, 100×.) (E) ^{59}Fe radioautograph of two BFU-E-derived colonies (200×). Silver grains almost completely obscure the benzidine-positive cells. (F) Cytocentrifuge preparation of a basophilic normoblast and a polychromatophilic normoblast harvested from plasma culture after 14 days of incubation with erythropoietin at 4 units/ml. (Benzidine–hematoxylin stain, 1300×.) (G) Cytocentrifuge preparation as in F. Fully hemoglobinized erythroid cell with a very pale staining nucleus together with an orthochromatic normoblast. (1300×)

RESULTS

Initial experiments involved plating the nucleated cell fraction isolated from peripheral blood by Ficoll–Hypaque density centrifugation in the plasma culture system. After 10–14 days of incubation at 37° in the presence of erythropoietin, macroscopic benzidine-positive colonies with characteristics similar to those of murine BFU-E-derived colonies were observed. The human erythroid colonies contained more than 100 cells and were composed of discrete subcolonies.

A low-power view (Fig. 1*A*) shows the appearance of these colonies in squash preparations permitting colony counts at a magnification of about 10×. Fig. 1*B* shows the absence of colonies in the absence of erythropoietin. Fig. 1*C* illustrates the morphology of BFU-E-derived colonies in the plasma clot at a magnification of 32×. The typical grouping of microscopic subcolonies within a macroscopic colony was thus visible both in culture (Fig. 1*C*) and in squash preparations (Fig. 1*D*). After 14 days of incubation cells were harvested from plasma culture and cytocentrifuge preparations were made. More than 20% of the cells observed were benzidine-positive and had the characteristic morphology of erythroid cells (Fig. 1*F* and *G*).

In a typical colony almost every cell stained intensely with benzidine–peroxide reagent. To further substantiate the presence of hemoglobin in the cells of each colony, ^{59}Fe incorporation followed by autoradiography was performed. A strong autoradiographic response was observed only in the areas of the emulsion directly over a macroscopic colony. A typical result is shown at a magnification of 200× in Fig. 1*E*. Development of erythroid colonies was also accompanied by synthesis of globin and globin mRNA (B. Forget, B. J. Clarke, and D. Housman, unpublished observation).

In Table 1 colony morphology has been quantitated as a function of time and dose of erythropoietin. Few colonies greater than eight cells are observed prior to 7 days of incubation. In a range of erythropoietin dosage of 1–4 units/ml the number of colonies and the time course of increase of benzidine-positive colonies were similar up to 13 days, when a plateau in colony number and proportion of large benzidine-positive colonies began. It should be noted that significant numbers of erythroid colonies appeared only in the presence of added erythropoietin. Between 16 and 18 days of incubation cultures began to deteriorate and extensive lysis of cells in erythroid colonies developed.

Very few mature granulocytic colonies developed in these cultures either in the presence or in the absence of erythropoietin. In all cultures containing erythropoietin, large numbers of benzidine-negative colonies were in evidence from day 7–9 (Table 1). However, with increasing incubation time, greater than 80% of the colonies became strongly benzidine-positive for heme and contained cells whose nuclear morphology was uniformly erythroid (Table 1, Fig. 1). Even with the highest concentration of erythropoietin employed (4 units/ml) and the longest incubation period (18 days), a residual 10–20% of the colonies remained benzidine-negative (Table 1). On a strictly morphological basis it was unclear whether these were immature granulocytic colonies or committed erythroid precursor cells which had not differentiated to the point of hemoglobin synthesis.

In Fig. 2 human and sheep erythropoietin are compared with respect to their ability to promote formation of macroscopic erythroid colonies. There was no significant difference in dose response between human and sheep erythropoietin at any concentration and a plateau of about 30 BFU-E-derived colonies per 5 × 10^5 nucleated cells plated was observed using erythropoietin at 1–4 units/ml. In some experiments a trend towards larger colony size at a higher erythropoietin dose was noted. For this reason a dose of 4 units/ml was chosen for subsequent experiments.

An analysis of colony size versus time of incubation is shown in Fig. 3. Average colony size increased from about 100 cells at day 7 to 350 at day 11–13. A moderate increase in colony size was observed between day 11 and day 16. The mean number of subcolonies per macroscopic colony also increased from a value of 3 at day 7 to maximum of 6 to 7 at day 13. On the basis of these results and those in Table 1, 13–14 days was used as an optimal incubation period for subsequent experiments.

For the assay of BFU-E from peripheral blood to have po-

Table 1. BFU-E-derived colony development in human peripheral blood cell cultures

Erythropoietin, units/ml	Days of incubation	Mean total no. colonies	Mean no. benzidine-negative colonies	Mean no. benzidine-positive colonies >100 cells	% benzidine-positive colonies
4	7	24	22	2	9
4	9	22	13	9	42
4	11	33	13	20	61
4	13	32	5	27	84
4	16	38	5	33	87
4	18	19	4	15	79
2	7	28	27	1	3
2	9	30	19	11	37
2	11	28	13	15	54
2	13	23	6	17	74
2	16	22	7	15	60
2	18	19	2	17	89
1	7	37	36	1	3
1	9	27	17	10	37
1	11	36	12	22	61
1	13	28	10	18	64
1	16	29	10	19	65
1	18	24	3	21	88
0.5	7	15	15	0	0
0.5	9	28	26	2	7
0.5	11	22	12	10	45
0.5	13	19	6	13	68
0.5	16	17	8	9	53
0.5	18	7	2	5	71
0	7	0	0	0	0
0	9	0	0	0	0
0	11	1	1	0	0
0	13	2	2	0	0
0	16	2	2	0	0
0	18	3	3	0	0

Data are based on three replicate cultures seeded at 3×10^5 nucleated cells per culture. Sufficient cells to perform the experiment were obtained by drawing 50 ml of blood from a single individual. Sheep erythropoietin was used in all experimental groups. A minimum colony size for benzidine-negative colonies was considered to be eight cells.

tential diagnostic value it must have a linear response with respect to cell input and a relatively narrow range of values when a series of normal subjects are assayed. Results suggesting that the assay meets these requirements are presented in Fig. 4 and Table 2. Fig. 4 shows that when cell input is varied between 10^5 and 6×10^5 cells per plate a linear dose response of macroscopic erythroid colony numbers is obtained. Below an input of 10^5 cells per plate the dose response becomes nonlinear, suggesting a possible requirement for a minimum number of input cells in the assay system. Table 2 illustrates the data compiled for six normal individuals assayed on six separate occasions. The values obtained ranged between 9.8 and 20.5 BFU-E-derived colonies per 10^5 nucleated cells plated with a mean value of 15.6 ± 2.1 BFU-E. One individual (B.C.) was assayed on three separate occasions over a period of six weeks and a mean value of 17.3 ± 5.7 BFU-E per 10^5 nucleated cells plated was observed. From this data and the total number of nucleated cells collected per ml of blood, it was calculated that normal individuals have approximately 100–200 BFU-E per ml of peripheral blood.

DISCUSSION

The dependence on a high concentration of erythropoietin, distinctive morphology, and large size of the erythroid colonies generated by cells present in human peripheral blood constitute strong evidence that these cells are analogous to murine BFU-E (3, 4, 8). The morphology peculiar to BFU-E-derived colonies is intrinsically interesting. Axelrad *et al.* (4) have hypothesized that the typical clustering of subcolonies observed in murine BFU-E-derived colonies is due to a period of motility during the differentiation of BFU-E to CFU-E. Because peripheral blood contains far fewer cell types than bone marrow, direct observation of human BFU-E cultures by time lapse cinematography may facilitate the characterization of this stage in the differentiation of BFU-E to the erythrocyte. As in the murine system, the *in vivo* role of the putative motile phase remains to be established.

Another important problem will be to establish cell surface characteristics of human BFU-E in order to obtain highly purified BFU-E populations. Immunological techniques for separating lymphocyte subsets have recently been employed to demonstrate that granulocytic precursor cells (CFU-C) found in human peripheral blood are localized in the null lymphocyte fraction (11). Results obtained by us in collaboration with C. Richman (unpublished) suggest that human peripheral blood BFU-E are also found primarily in the null lymphocyte subset.

Our observations raise a significant question about the role of peripheral blood BFU-E in normal erythropoiesis. Do these

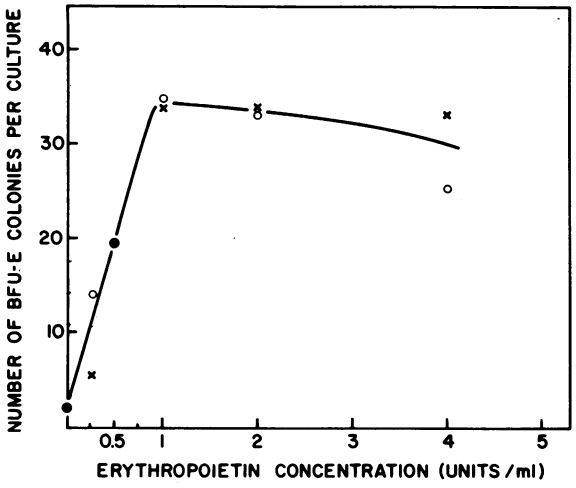

FIG. 2. Relationship between the number of BFU-E-derived colonies per culture after 13 days of incubation and dose of either sheep (×) or human (○) erythropoietin. Nucleated cells from the peripheral blood of one individual were plated at a concentration of 5×10^5 cells per culture. Data points are the means of three replicate cultures.

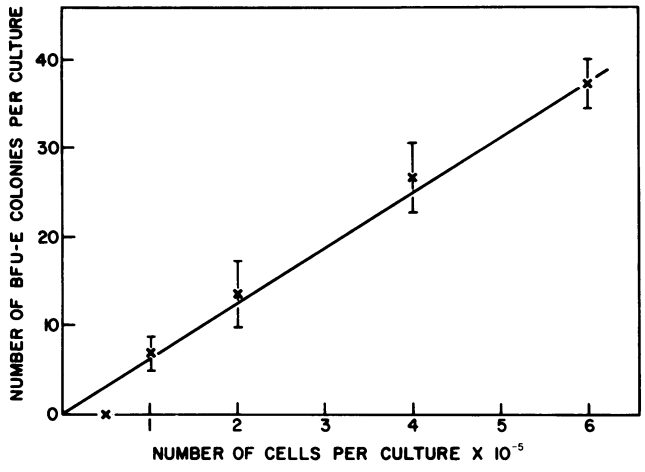

FIG. 4. Correlation between the number of BFU-E-derived colonies observed and nucleated cells plated per culture. Peripheral blood nucleated cells from a single individual were plated in plasma cultures containing sheep erythropoietin at 4 units/ml. Data points represent the mean ±SEM number of BFU-E-derived colonies per culture after 11 days of incubation.

cells simply represent an overflow from the marrow into the peripheral blood or could they be an important vehicle for redistributing erythropoiesis from one site in the marrow to another? One striking aspect of our results is that the erythroid precursor cells from peripheral blood appear to exhibit a higher proliferative capacity than has yet been reported for cells from human marrow (6, 7). Preliminary experiments in our laboratory suggest that bone marrow cells plated under identical experimental conditions give rise to smaller erythroid colonies than peripheral blood cells. These observations raise the question of suppressor cells in the marrow population. Heath *et al.* (8) were unable to demonstrate the presence of such cells in murine marrow on the basis of experiments in which the marrow cell population was fractionated according to size. Appropriate cell separation experiments as well as mixing experiments between marrow and peripheral blood cells should help to resolve these issues in the human system.

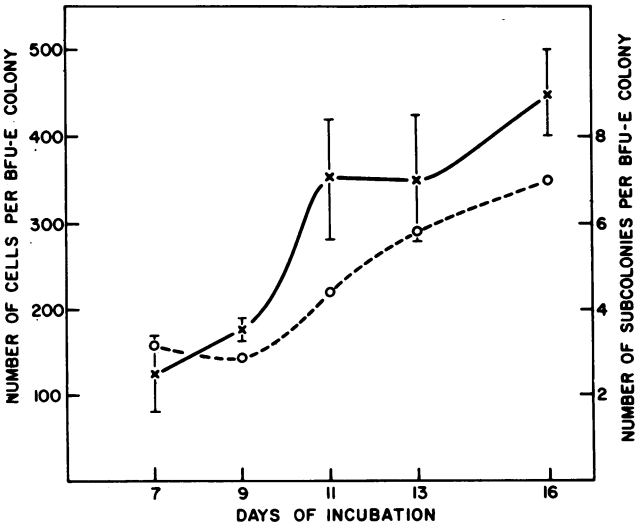

FIG. 3. Development of BFU-E-derived colony size with increasing time in culture. Data for both mean ±SEM cell number per BFU-E-derived colony (×) and the mean number of subcolonies per BFU-E-derived colony (○) is based on a count of 15 BFU-E-derived colonies per time point in cultures containing 3×10^5 nucleated cells and 4 units/ml sheep erythropoietin.

The presence of an erythroid precursor cell of high proliferative capacity in human peripheral blood is of potential significance to a number of approaches to human erythropoiesis. The ability to cultivate a relatively synchronous cohort of normal erythroid precursor cells *in vitro* should facilitate biochemical analysis of early events in human erythropoiesis. For example, the routine availability of early erythroid precursor cells should facilitate study of the switch from adult to fetal hemoglobin synthesis *in vitro* (12). We have found that globin synthesis in the thalassemia syndromes can now be studied using peripheral blood precursor cells rather than cells from marrow aspirates. Globin chain production by peripheral blood BFU-E-derived erythroid colonies from a number of thalassemia patients clearly demonstrates imbalance of globin chain synthesis similar in magnitude to that observed in reticulocyte lysates from the same individuals (B. Forget, B. J. Clarke, and D. Housman, unpublished observations). Our observations also appear to be relevant to clinical situations involving other disorders of erythropoiesis. Recent results from our laboratory have indicated that three patients with Blackfan–Diamond anemia had no detectable BFU-E in the peripheral blood before ameliorating steroid treatment began (13). Once in clinical remission

Table 2. Numbers of BFU-E in the peripheral blood of normal individuals

Name, sex	Mean no. BFU-E-derived colonies
B.C., male	14.7 ± 0.4
D.H., male	10.3 ± 1.5
B.M., male	20.3 ± 4.3
D.N., male	9.8 ± 1.8
J.L., female	18.0 ± 2.9
L.S., female	20.5 ± 0.5
Mean ± SEM	15.6 ± 2.1

Data are based on three replicate cultures incubated for 13 days. All cultures contained 10^5 nucleated cells and 4 units/ml sheep or human erythropoietin. Individuals ranged in age from 25 to 45 years.

the same patients had detectable but abnormally low numbers of BFU-E in their blood (13). It therefore appears that the frequency of erythroid precursor cells in peripheral blood will be an important parameter in the diagnosis and monitoring of therapy in disease states affecting erythropoiesis.

While this manuscript was in preparation we learned of similar work by Ogawa and coworkers (14) using a different technique for assaying erythroid precursor cells, cloning in methylcellulose. Our results are in quantitative agreement with respect to numbers of precursor cells in human peripheral blood, although quantitation of colony size and morphology is more difficult in the methylcellulose system and was not reported by these workers. It will clearly be of interest to further compare the behavior of peripheral blood erythroid precursor cells in the two experimental systems.

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