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Evidence for structured variation in self-renewal capacity within long-term bone marrow cultures

(serial transplantation/stem cell proliferation/hematopoiesis)

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ABSTRACT **Bone marrow pluripotent stem cells (CFUs) demonstrate capacity for both proliferation and differentiation. The proliferative capacity of CFUs has been measured by serial transplantability and by the R_s, a measurement of CFU production in a single 14-day transfer. In the present study, the self-renewal capacity of both adherent and nonadherent CFUs from long-term bone marrow cultures was measured. Culture conditions were established such that nonadherent cells were derived from the adherent cell layer. Both adherent and nonadherent cells produced spleen colonies, demonstrating that significant proliferative potential was present in both locations; however, at all times in culture, the CFUs within the adherent stromal cell layer had a significantly greater self-renewal capacity than did the nonadherent CFUs. During the initial establishment of the cultures, the self-renewal capacity of the adherent CFUs decreased as the total number of CFUs per flask increased. After 3 weeks in culture, the self-renewal potential of the adherent CFUs stabilized and was maintained. These results suggest two different mechanisms of stem cell proliferation. In order to increase the most primitive stem cell pool size, there was initial proliferation of early stem cells with a concomitant decrease in self-renewal capacity. Once this pool was established, the self-renewal capacity of the adherent CFUs maintained for 13 weeks in culture suggests that CFU production and cell maintenance were achieved by clonal succession.**

The pluripotent hematopoietic stem cell (CFU) is the most primitive of the known hematopoietic progenitor cells. A standard assay for this cell described by Till and McCulloch (1) measures the ability of donor marrow cells to form colonies in the spleens of lethally irradiated syngeneic mice. The self-renewal capacity of the CFUs has been measured by both the R_s, a measurement of CFU production over 14 days of serial marrow transplantation, and by the maximal transplantation time in a series of passages in irradiated animals (2). The standard CFU assay cannot distinguish stem cells with different self-renewal capacities because all produce spleen colonies. By assaying marrow from mice long after the administration of certain alkylating agents, a decrease in the self-renewal potential of the CFU compartment has been demonstrated *in vivo* (2–5). These experiments are consistent with the notion that the CFU compartment is heterogeneous and consists of a continuum of cells varying in self-renewal capacity. Within this continuum, early CFUs would be less committed to differentiation and have a high self-renewal capacity whereas CFUs further along in the continuum would be more committed to differentiation and have a lower self-renewal potential. Busulfan and certain other alkylating agents would preferentially destroy cells early in the continuum (2, 3). Heterogeneity within the CFU compartment has also been measured *in vitro* (6, 7). With selective inactivation of the CFUs by rabbit anti-mouse brain antiserum, Monette (6) has found a greater self-renewal capacity in the remaining nonaffected CFUs. Similarly, Worten *et al.* (7) have separated bone marrow by sedimentation velocity and found CFUs with a high self-renewal capacity in the slowly sedimenting fraction.

In the study reported here, we used a long-term bone marrow culture system to study the nature of proliferation and maintenance of the CFU pool. These experiments were designed to study the self-renewal potential of the CFU *in vitro* both as the culture is established and then as it is maintained in a steady state. Specifically, we were interested in whether or not self-renewal capacity decreased with time in culture during either of these phases of *in vitro* proliferation.

MATERIALS AND METHODS

Bone Marrow Cultures. Male C3H/HeJ mice (The Jackson Laboratory) 8–10 weeks old were sacrificed by cervical dislocation and the marrow content of a single femur and tibia (approximately 2.0×10^7 cells) was flushed with 10.0 ml of Fischer's medium (GIBCO) supplemented with 25% donor horse serum (Flow Laboratories, McLean, VA) and 10 μM hydrocortisone sodium hemisuccinate (SoluCortef, Upjohn) into 25-cm^2 plastic flasks (Corning). Cultures were then incubated at 33°C in room air with 7% CO_2. Weekly, all nonadherent cells were removed and the medium was replaced with 6 ml of fresh medium and 4 ml of cell-free culture medium from the previous week.

Representative flasks were harvested weekly, and cells taken from both the adherent and nonadherent layers were assayed separately. Flasks were washed twice with 5.0 ml of fresh medium; after gentle agitation the washings were pooled with the nonadherent fraction. Adherent cells were detached from the flask with a rubber policeman, and single-cell suspensions were obtained by passage through a 22-gauge needle. Cells were pelleted at $450 \times g$ for 7 min and resuspended in fresh medium for tail vein injections. The contents of at least 10 flasks were pooled for each serial transfer of marrow, and at least 3 flasks were pooled for each CFU measurement.

The following modifications in the Dexter technique (8, 9) of marrow culture were made: a single inoculum of marrow was cultured in hydrocortisone-supplemented media (10, 11); cultures were not recharged with fresh bone marrow (12); and all nonadherent cells were removed each week so that subsequent nonadherent cells were known to be derived from the adherent cell layer. Because bone marrow was introduced only at the initiation of the culture and all nonadherent cells were removed weekly, the provenance of cells in each location could be determined.

Self-Renewal Assay for CFUs. Recipient mice received 1250 rad (12.50 grays) from a ^{137}Cs source in doses of 700 and 550 rad 3 hr apart through opposing portals for both serial transfer and CFU determination (2). Serial transplantation was done at 14-day intervals. At each transfer, similarly prepared re-

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Abbreviation: CFU, pluripotent stem cells (colony-forming units).

cipient animals were injected with appropriate number of cells and 8 days later were sacrificed for spleen colony determination by the method of Till and McCulloch (1). Approximately 100 CFUs were injected into each of 10 animals for the first transfer and a total of 1×10^6 bone marrow cells were injected per mouse for subsequent transfers. The total time of serial transfer was recorded as the maximal survival time of any recipient. For example, if all 10 animals survived the first 14 days, 7 animals the next 14 days, and the last recipient of the third transfer died after 10 days, then the maximum transfer time would be 14 + 14 + 10 or 38 days.

The proliferative capacity of the adherent and nonadherent cells also was assayed by an alternate method for measuring self-renewal capacity. This proliferative capacity (R_s) can be expressed by the ratio of number of marrow CFUs produced in a single transfer to number of those inoculated. This has been described (2, 3). It is calculated by $R_s = S_n/KS_i$ in which S_i is the number of CFUs injected, S_n is the number of CFUs measured in the hind limb after 14 days of proliferation, and K is a correction factor assumed for the proportion of CFU seeding the marrow as compared to other hematopoietic sites.

RESULTS

Nucleated Cell and CFU Measurements in Long-Term Bone Marrow Cultures. The numbers of nucleated cells per flask determined weekly are recorded in Table 1. The number of adherent cells gradually increased over the first 5 weeks in culture and then stabilized (all nonadherent cells were removed weekly). The numbers of adherent and nonadherent CFUs per culture assayed weekly also are shown in Table 1. At 1 week, only $7.3 \pm 1.2 \times 10^5$ cells remained attached to the flask surface and only 15 ± 1 adherent-layer CFUs were detected per flask. Because all nonadherent cells were removed at the first medium change, the remaining adherent cells provided for the proliferation noted over the next 13 weeks in the culture system. Between the first and third weeks in culture there was a rapid increase in the number of CFUs per flask, initially noted in the adherent cell layer and followed shortly by an increase in the number of nonadherent CFUs. The large number of CFUs detected at week 3 corresponded with the appearance of fat-containing cells in the adherent layer. After the fifth week in culture, the total number of adherent and nonadherent CFUs per culture gradually declined, but there continued to be 43–205 adherent CFUs and 13–140 nonadherent CFUs out to 13 weeks. All nonadherent cells were removed weekly, so subsequent nonadherent cells were derived from the adherent cell layer.

Table 1. Weekly cell counts and CFU determination in long-term bone marrow cultures

Weeks in culture	Adherent layer: Nucleated cells × 10^{-5} per flask	Adherent layer: CFUs per flask	Nonadherent layer: Nucleated cells × 10^{-5} per flask	Nonadherent layer: CFUs per flask
1	7.3 ± 1.2	15 ± 1	51.6 ± 9.6	133 ± 29
2	29.1 ± 3.5	107 ± 22	20.8 ± 2.9	45 ± 15
3	34.8 ± 4.6	245 ± 61	9.1 ± 1.0	183 ± 32
4	40.3 ± 8.8	325 ± 56	13.0 ± 5.6	124 ± 46
5	57.5 ± 1.1	273 ± 59	37.9 ± 14.1	153 ± 31
6	55.1 ± 9.4	158 ± 65	33.8 ± 15.5	71 ± 11
7	64.2 ± 0.4	158 ± 121	28.3 ± 9.0	72 ± 19
8	40.5 ± 1.7	88 ± 12	26.9 ± 4.0	61 ± 16
9	34.4 ± 11.0	89 ± 53	12.1 ± 2.8	34 ± 18
10*	42.0	102	13.2	41
11	62.8 ± 10.9	141 ± 24	43.7 ± 6.3	53 ± 16
12†	72.2	43	17.8	13
13*	59.0	205	44.0	140

At least three flasks were harvested and pooled for each cell count and CFU determination. Separate experiments were performed for each week in culture. Mice were sacrificed for CFU determination 8 days after lethal irradiation and tail vein injection. Results are shown as mean ± SEM.

* Two separate experiments were performed.

† One experiment was performed.

Self-Renewal Capacity of CFUs in Continuous Bone Marrow Cultures. Adherent and nonadherent CFUs were separately injected for serial transplantability and R_s determination after various times in culture (Table 2). After inoculation of bone marrow into tissue culture flasks, there appeared to be selective attachment of CFUs with a high self-renewal potential to the flask surface. The adherent-cell R_s was 92.7 ± 23 at 6 days in culture just prior to the initial medium change; the nonadherent-cell R_s was 24.7 ± 4.6. Both adherent and nonadherent cells from 6-day cultures had a maximal transplantation time of 42 days. At the first medium change, approximately 10 nonadherent CFUs were removed per adherent CFU remaining.

Between the first and third weeks in culture there was a large increase in nucleated cells and CFU production. From the 15 ± 1 adherent layer CFUs noted at week 1 there was an increase to 428 adherent and nonadherent CFUs per flask at week 3 (Table 1). Over this same time interval, the mean (± SEM) R_s of the adherent CFUs declined from 92.7 ± 23 at day 6 to 9.7 ± 3.5 at week 3 (Fig. 1). This decline in R_s then paralleled the initial increase in CFUs per culture.

The self-renewal potential of the adherent CFUs remained constant from week 3 to week 13 as measured by R_s and serial transplantability (Fig. 1; Table 2). Regression curve analysis for the R_s data revealed a horizontal slope with no decline in R_s over this time interval. Similarly, the mean maximal transplantation time of adherent cells remained constant from week 3 to week 13 after the initial decrease. During weeks 3–5, the number of nonadherent CFUs produced and removed weekly remained relatively constant and then began to decrease. However, a substantial number of adherent and nonadherent CFUs were found weekly out to the 13 weeks in culture assayed (Table 1). Due to gradual deterioration of the stromal cell layer in 10- to 16-week-old cultures maintained in medium supplemented with horse serum, longer times in culture were not assayed.

Adherent and nonadherent CFUs from 3 to 8-week-old cultures were assayed separately for serial transplantability and R_s (Table 2). In each of 16 experiments the R_s of the adherent CFUs was greater than the R_s of nonadherent CFUs. Cells from the adherent layer had a mean R_s of 8.9 compared with a mean R_s of 0.7 for nonadherent CFUs ($P < 0.001$, t test). These differences in R_s were also reflected in serial transplantability. In 10 of 16 experiments, mice injected with adherent cells survived at least through the end of the second transfer in contrast to a second transfer survival in only 1 of 16 experiments when nonadherent CFUs were injected. The mean maximal transplantation time from week 3 to week 13 in culture was 28 days for adherent cells and 20 days for nonadherent cells ($P = 0.02$, t test).

DISCUSSION

The present study attempted to define differences in self-renewal potential in a long-term bone marrow culture system. We have shown marked differences in self-renewal potential between adherent and nonadherent CFUs, and between adherent CFUs from short- and long-term cultures as measured by both R_s and serial transplantability. Because both adherent and nonadherent CFUs produce indistinguishable macroscopic spleen colonies, it appears that nonadherent CFUs also have significant proliferative capacity.

Table 2. Self-renewal capacity of adherent and nonadherent CFUs from long-term bone marrow cultures assayed over 13 weeks

Weeks in culture	Experiments, no.	R_s		Maximal transplantation time, days	
		Adherent cells	Nonadherent cells	Adherent cells	Nonadherent cells
1	3	92.7 ± 23	24.7 ± 4.6	42	42
2	3	20.3 ± 6.4	2.2 ± 0.4	37	19
3	4	9.7 ± 3.5	1.7 ± 1.3	21	24
4	5	8.4 ± 4.5	0.3 ± 0.2	34	14
6	3	10.9 ± 4.5	0.3 ± 0.2	28	22
8	4	7.4 ± 1.9	0.3 ± 0.2	30	21
11	2	9.8	—	28	—
13	2	14.7	—	28	—

At least 10 flasks were pooled for each determination of R_s and maximal transplantation time. Separate experiments were performed for each week in culture. Approximately 100 CFUs were injected into lethally irradiated mice for the first transfer and a total of 1×10^6 bone marrow cells were injected for each subsequent transfer. Similarly prepared animals were injected with appropriate numbers of cells and sacrificed 8 days later for spleen colony determination. Results are shown as mean ± SEM.

At all assay times the adherent CFUs had a greater R_s than did the nonadherent CFUs. After initiation of cultures, CFUs with a high self-renewal capacity preferentially appeared to attach to the flask surface, whereas a larger portion of CFUs of near-normal self-renewal potential remained unattached. An alternative explanation is that initial attachment confers on cells a greater capacity for self-renewal. A weighted average R_s was calculated by multiplying the proportion of CFUs by the mean R_s for adherent and nonadherent cells at day 6 (Table 3). The calculated mean R_s of 31.6 is within the range of normal bone marrow (20–40) and supports the notion that selective attachment of CFUs of high self-renewal capacity occurs rather than environmental alteration of CFUs after attachment. After initial removal of all nonadherent cells at day 6, subsequent nonadherent CFUs, which must have arisen from the adherent cell layer, had a low R_s and a decreased mean maximal transplantation time. From the present data, an association exists between adherence of CFUs and high self-renewal capacity. This high self-renewal capacity is maintained in the long-term bone marrow cultures and is associated with the continued weekly production of CFUs. As cells became detached they lost the capability for self-renewal. The factors determining detachment are not known. Detachment and loss of cell-to-cell interaction may stimulate the change in self-renewal capacity or, alternatively, loss in self-renewal potential and perhaps commitment to differentiation may promote the cellular membrane changes that favor detachment. Several studies now confirm that the maintenance of CFUs in long-term bone marrow cultures is dependent on the integrity of an adherent cell microenvironment including macrophages, epithelial cells, and giant lipid-containing cells (8, 13, 14). The nature of the cell-to-cell contact required for maintenance of CFUs in long-term cultures is unknown.

The present study has defined four phases of bone marrow growth *in vitro*: attachment, initial proliferative phase, steady state, and decline. Preferential attachment to the flask surface of a small number of CFUs with high self-renewal potential occurs during the first week of culture. Initial proliferation of the CFU compartment occurs during the first 3 weeks of culture. From week 1 to week 3 there was a 30-fold increase in the

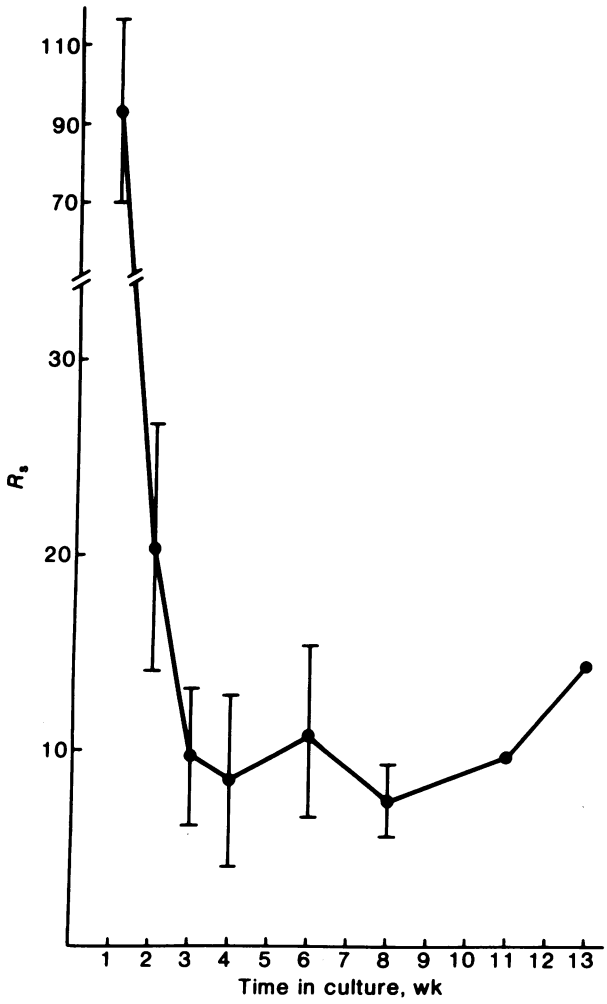

FIG. 1. Self-renewal capacity of CFUs in the adherent cell layer of long-term bone marrow cultures. The R_s was determined for adherent cell harvests at each indicated time point. The results represent the mean ± SEM. The total number of experiments performed is outlined in Table 2.

Table 3. Selective attachment of CFUs with high self-renewal capacity in tissue culture flasks assayed at 6 days

	CFUs per flask	R_s
Adherent cells	15	92.7
Nonadherent cells	133	24.7
Total	148	31.6*

* R_s determined by: $\overline{R}_s = (R_S\text{-CFU}/\Sigma\text{CFU})$ for adherent cells + $(R_s\text{-CFU}/\Sigma\text{CFU})$ for nonadherent cells = (92.7 × 15/148) + (24.7 × 133/148) = 31.6. For normal bone marrow, $\overline{R}_s$ = 20–40.

number of CFUs per culture and a simultaneous 90% decrease in the self-renewal capacity of the adherent CFUs as measured by R_s. This early decrease in R_s may be analogous to the development of fetal marrow in which self-renewal potential initially is high and then decreases as the gestational age increases (15). This decrease occurs presumably because proliferation of primative CFUs results in a loss of limited proliferative capacity as described (16). Similarly, busulfan causes a permanent decrease in R_s (2, 3). It preferentially damages CFUs with the greatest self-renewal capacity, requiring the remaining CFUs to repopulate the CFU compartment and resulting in a decrease in R_s. Similar changes in R_s occur with transplantation of small numbers of CFUs into irradiated recipients (2, 17). Thus, whenever the early CFU pool is small and requires expansion, there is proliferation of such cells and their self-renewal decreases.

From week 3 to week 13 in culture, the number of CFUs per flask was initially constant and then slowly decreased. The R_s of adherent CFUs remained constant throughout this period. Hayflick (16) has observed that normal diploid cells have only a limited proliferative capacity *in vitro*. To reconcile Hayflick's observation with rapidly proliferating normal cell renewal systems *in vivo*, Kay (18) proposed a theory of clonal succession, later amplified by Reincke *et al.* (19, 20). This model suggests that most CFUs are held in reserve, the marrow being supplied by a limited number of CFUs which, when exhausted, stimulate active proliferation of new CFUs. The clonal succession theory would predict that the adherent cell R_s would remain constant with time in culture although the absolute number of CFUs would gradually decrease as more reserve cells enter active proliferation. The *in vitro* data presented for weeks 3 to 13 are consistent with this theory. Similarly, R_s and serial transplantability is maintained in old mouse marrow and in animals treated with 5-fluorouracil, a cycle active agent (3, 21). Stimuli that increase end-cell demand such as bleeding, high altitude, and cycle active agents do not result in a decrease in the self-renewal of the CFU compartment.

It appears that after approximately 15 weeks in culture there is deterioration of the stroma and variable loss of CFUs in the cultures. Whether deterioration of the culture is due to death of adherent stroma cells or exhaustion of the CFU pool is unknown at present.

A model for the evolution of the stem cell which accounts for the findings of the *in vitro* culture system and also explains bone marrow growth *in vivo* is shown in Fig. 2. This model suggests different hierarchical mechanisms used as CFUs meet different circumstances. When a few CFUs with a great capacity for self-renewal are required to give rise to a greatly enlarged CFU compartment, all CFUs divide relatively uniformly, thus decreasing the average self-renewal capacity. During this time period, CFUs have a uniform generational age: all CFUs present are subject to proliferative stress and cell division. This process is seen *in vitro* during the first 3 weeks of culture, *in utero* (15), after bone marrow transplantation (2, 17), and after exposure to agents that destroy early CFUs (2, 3, 22). Once there is adequate expansion of the CFU compartment, a steady state is reached. End-cell proliferation requires only limited CFU activity, and clonal succession obtains. CFUs are recruited as needed for this maintenance without continued proliferative activity of the pool, and thus the self-renewal capacity remains constant. This is seen *in vitro* during maintenance of bone marrow cultures and during the life-span of the mouse (2). Even stimuli that increase mature cell demand such as bleeding, high altitude, and cycle active agents require little compensatory activity of the most primitive CFUs. This model reconciles a limited proliferative capacity of the CFUs with all known phases of *in vitro* and *in vivo* growth as well as to the response to various external agents. Studies with long-term bone marrow cultures may better define the limitations of the hematopoietic CFU and its microenvironment.

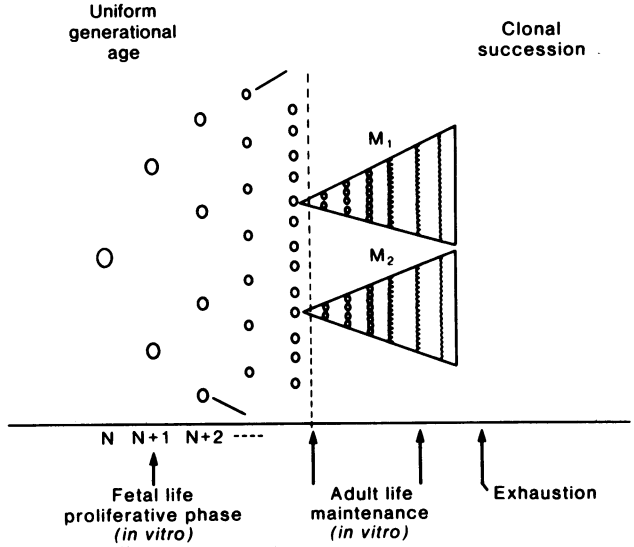

FIG. 2. Model for hematopoiesis to reconcile *in vitro* and *in vivo* findings.

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