The Effect of Stem Cell Proliferation Regulators Demonstrated With an in vitro Assay


Spleen colony formation after transplantation of bone marrow cells into irradiated mice has been used as an assay for hematopoietic stem cells (CFU-S), but has serious limitations intrinsic to an in vivo assay. In this report we describe experiments using an in vitro clonogenic assay that is especially suitable for studies of stem cell regulation as defined growth factors and normal untreated bone marrow can be used. We have demonstrated that the colony-forming cells have proliferative properties in common with CFU-S and respond to specific proliferation regulators previously detected using the spleen colony assay.

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MATERIALS AND METHODS

Mice

Normal bone marrow was obtained from 8- to 12-week-old male NIH-Ola or female B6D2F1 mice. To obtain regenerating bone marrow (PH-BM) B6D2F1 mice were treated with phenylhydrazine\(^*\), 60 mg/kg was injected subcutaneously seven, six, and four days previously. No significant differences between the two strains were detected and the data are reported together.

Cell Lines and Conditioned Media

We used the L-929 fibroblast cell line\(^*\) as a crude source of CSF-1 (M-CSF) in our studies. Cells were grown in roller bottles with modified Eagles medium (MEM) and 10% fetal calf serum to half-confluence. Spent medium was then removed, replaced with fresh medium as above, and the cultures were allowed to grow for three more days. The conditioned medium (L929-CM) was removed, passed through 0.22 \(\mu\)m filters and stored at \(-20^\circ\)C. Our source of activity synergizing with L929-CM to give macroscopic colonies was the cell line AF1-19T, which is an NRK (rat fibroblast) cell line transformed with the malignant histiocytosis sarcoma virus (MHV).\(^*\) Conditioned media from this cell line (AF1-19T-CM) was prepared as for L929-CM.

Clonogenic Assays

CFU-S

CFU-S were assayed by injecting 0.2 mL aliquots containing 5 to 8 \(\times 10^6\) cells into 8.2 Gy x-irradiated mice (ten per group).\(^*\) Spleen colonies were counted on day 12. The seeding factor (f) for the CFU-S was determined as published previously.\(^*\) Where indicated, the experimentally determined f factor was 0.053.

GM-CFC

GM-CFC were assayed by culturing 1 to 5 \(\times 10^6\) cells in 30-mm petri dishes containing 0.3% agar in Fischer's medium supplemented with antibiotics, 2 mmol/L glutamine, and 20% horse serum. Conditioned media from L929 cells\(^*\) and the malignant histiocytosis sarcoma virus-transformed cell line AF1-19T\(^*\) were used as sources of colony stimulating activity. The 1-mL cultures were incubated at 37\(^\circ\)C in a fully humidified atmosphere of 5% CO\(_2\) in air and colonies were counted on day 7.

Primitive Progenitors

CFU-A (colony-forming units type A). For the detection of primitive progenitors 10^6 cells in 4 mL supplemented alpha-modified MEM containing 25% fetal calf or horse serum and 0.3% agar were seeded on top of an underlayer of the same medium containing 0.6% agar, 10% L929 cell conditioned medium (L929 CM, a source of CSF-1), and 10% AF1-19T cell conditioned medium (AF1-19T CM) in a 6-cm petri dish. Cultures were incubated at 37\(^\circ\)C in a fully

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humidified atmosphere of 10% CO₂, 5% O₂, and 85% N₂ for 11 days. Colonies were stained with INT (2-(4-iodophenyl)-3-(4-nitropheno-nyl)-5-phenyltetrazolium chloride) overnight. While there are colonies in the CFU-A assay with a diameter < 2 mm, we have chosen this value as a useful cut-off point after preliminary experiments were performed using cytosine arabinoside (see below). We found that within individual dishes, colonies with a diameter < 2 mm were mainly derived from cells in cycle, whereas colonies > 2 mm were found to be derived from minimally cycling cells. Only colonies with diameters > 2 mm were scored in these assays.

In some experiments purified or recombinant growth factors were used. CFU-A assays were set up as described above except that AFI-19T CM and L-929 CM were substituted with recombinant murine GM-CSF and purified CSF-1, respectively. Recombinant murine GM-CSF (Biogen, Geneva) was used at an optimal concentration of 400 pg/mL culture and CSF-1 at a concentration of 131 U/mL. In experiments with antiserum preparations, CSF-1 antiserum (kindly provided by Dr. E.R. Stanley, New York) was added directly on initiation of cultures at a dilution (1:400) previously determined by titration to be sufficient to prevent significant colony formation in the assay. Suboptimal amounts of AF1-19T CM (5% in each case) were used in these experiments. IL-1α antiserum (Biogen, Geneva) was also added directly to the cultures at a dilution (1:1,000) that we had previously determined to inactivate IL-1α activity in the HPP-CFC assay. In these experiments recombinant murine GM-CSF was used at a suboptimal concentration of 109 pg/mL culture and L-929 CM was used at a suboptimal level of 5%.

**Replating Experiments**

Individual seven-day colonies (average, 4 x 10³ cells) were plucked from cultures set up as described above, using L929 CM and AF1-19T CM as growth factors, except that 0.9% methocel was used in the upper layer instead of 0.3% agar. Plucked colonies were transferred to small tubes, one colony per tube containing 100 μL L929 CM and 100 μL AF1-19T CM. The tubes were agitated gently to obtain a single cell suspension, 1 mL of 0.9% methocel in α-MEM with 25% horse serum was added and the contents of each tube was transferred onto a 1-mL layer of 0.6% agar in α-MEM in a 3-cm dish. The dishes were incubated for 11 days. The secondary macroscopic colonies visible at day 11 were stained with INT. Colonies > 1 mm in diameter were scored as CFU-A in these replating experiments since previous experiments had shown that in the standard CFU-A assay, average colony diameters were smaller with methocel as the semi-solid support in otherwise identical conditions compared with the standard soft-agar conditions described above.

**The Kinetics of Recovery After 5-Fluorouracil Treatment**

Groups of five NIH-Ola mice were given a single dose of 5-fluorouracil (5-FU) at 150 mg/kg.8,18 Bone marrow was obtained at different times after treatment and the content of CFU-A, CFU-S, and GM-CFC was measured as described above. For CFU-S assays recipient NIH-Ola mice (ten per group) received 9 Gy (900 rad). CFU-S were measured 12 days after inoculation with donor marrow cells. CFU-S values are not adjusted with the seeding factor (f). Noninoculated control animals displayed a background of less than one colony per spleen. GM-CFC were measured as described above using 437 pg/mL of murine recombinant GM-CSF.

**Preparation of CFU-S Proliferation Factors**

The procedure of Lord et al was followed. Preparations of CFU-S proliferation inhibitor and stimulator were prepared from normal bone marrow and regenerating bone marrow (B6D2F1 mice), respectively.12,13

**Assessment of Cycling Status**

A suicide technique was used for the assessment of the proliferative status of the various clonogenic cells. Bone marrow was incubated in pairs of tubes containing 5 x 10⁶ cells in 1 mL Fischer's medium supplemented with 20% horse serum. Either 50 μg/mL CFU-S proliferation inhibitor or 250 μg/mL CFU-S proliferation stimulator or α-MEM was added to each tube and Fischer's medium was added to control tubes. The mixtures were incubated at 37°C for five hours (inhibition assays) or two hours (stimulation assays). For the last 60 minutes of the incubation 10⁻³ mol/L cytosine arabinoside was added to one tube and an equal volume of medium to the other tube. Cells were then washed twice before being assayed. The proliferation factors have no toxic effect on CFU-S, CFU-A, GM-CFC, or total nucleated cells, and have been shown to be CFU-S specific.11

**RESULTS**

**Macroscopic Colony Formation**

Bone marrow cells plated out in semi-solid agar in the presence of L929 CM and AF1-19T CM gave rise to macroscopic colonies after 11 days in culture (Fig.1A). The colonies ranged in diameter from 2 to 5 mm and contained an average of 4.5 x 10⁶ cells. There was a variable number of macroscopic colonies with diameters < 2 mm. We have chosen this value as a useful cut-off point (see Materials and Methods). The linear response of colony formation to the number of cells plated (Fig.1B) suggests that one cell is limiting and that it is likely that the line shown indicates the frequency of the cell of origin of the colony. In a series of experiments, we determined for normal bone marrows an average frequency of 197 ± 22/10⁶ NIH Ola, 145 ± 16/10⁶ BD26F1, and 180 ± 14/10⁶ BALB/c. Most colonies were composed primarily of macrophages with a small proportion of granulocytes and occasionally immature erythroid cells at day 11 of the assay. These observations prompted an analysis of development of colonies in the assay. We found that between days 6 and 9 of culture an average of >80% of these developing colonies (macroscopic colonies with an average cell content of 4 x 10⁶ cells) stained in situ with benzidine and cytospin preparations of these colonies contained variable proportions of erythroid, granulocytic, megakaryocytic, macrophagic, and blast cells. Mapping experiments in which development of typical large colonies at days 6 to 9 was observed in situ revealed that the majority developed into colonies with >2 mm diameter. No macroscopic colonies > 2 mm developed in dishes after plucking at days 6 to 9. In a typical experiment with B6D2F1 bone marrow cells we observed a ratio of 2.4:1 of macroscopic colonies (> 2 mm) to smaller colonies (< <2 mm). Since there is varying background colony formation (<0.5 mm) due to the L929 CM alone we assume that the colonies < 2 mm may be derived from a heterogeneous population of cells, which would include both primitive and more mature progenitor cells.

**The Mixed Colony Assay**

The procedure of Lord et al was followed. Preparations of CFU-S proliferation inhibitor and stimulator were prepared from normal bone marrow and regenerating bone marrow (B6D2F1 mice), respectively.12,13
Table 1. Replating of Macroscopic Colonies

<table>
<thead>
<tr>
<th>Colonies plucked</th>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original colonies forming 2+ colonies</td>
<td>63</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Replating efficiency (%)</td>
<td>4.8</td>
<td>60</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>2+ colonies &gt;1 mm</td>
<td>3</td>
<td>26</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>&lt;1 mm</td>
<td>437</td>
<td>168</td>
<td>441</td>
<td></td>
</tr>
<tr>
<td>Percent of cells in 1+ colonies forming 2+ colonies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 mm</td>
<td>0.17</td>
<td>0.21</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>&gt;1 mm</td>
<td>0.001</td>
<td>0.03</td>
<td>0.048</td>
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</tbody>
</table>

CFC colonies (approximately 500 to 1,000 cells per colony) although their ability to take up the INT dye suggests that the cells are still viable at day 11 in the assay. In contrast, GM-CFC colonies stimulated with recombinant GM-CSF alone show variable staining in the day 7 GM-CFC assay. In parallel experiments (data not shown) these GM-CFC colonies plucked at day 7 showed no ability to replate when stimulated with AF1-19T CM plus L929 CM with only a few clusters of cells being detected. Primary day 11 CFU-A colonies do not have any significant capacity to form secondary macroscopic colonies.

In preliminary experiments we have also tested the developing day 5 CFU-A primary colonies for the presence of CFU-S. Colonies at day 5 of the assay were plucked and pooled before injection into lethally irradiated mice (see Materials and Methods). We found in two separate experiments that there was an average of six day 12 CFU-S per colony assuming a seeding factor (see Materials and Methods) previously determined for normal bone marrow CFU-S. More experiments will be required to determine the optimum CFU-S content during macroscopic colony (CFU-A) growth. The results described above show that CFU-A have the capacity for self-renewal consistent with their being primitive in nature and similar to CFU-S.

The Effect of Cytotoxic Drug Treatment

To compare characteristics of the CFU-S and CFU-A we studied the response of these two cell types to cytotoxic drug treatment in vivo. It has been shown that a single injection of 5-FU results in an early ablation of committed progenitor and stem cells in the mouse. In response to this stress the progenitor cells recover to normal values within seven to ten days while the stem cells (CFU-S) show a marked overshoot between days 10 and 14 and have returned almost to normal levels by day 18. These differences in the kinetics of recovery allow us to distinguish between progenitor cells and more primitive cells like CFU-S. In light of the similarity between CFU-A and CFU-S we measured CFU-S, CFU-A, and granulocyte-macrophage progenitor cell (GM-CFC) numbers in the bone marrow at different times after a single injection of 5-FU (Fig 2). The CFU-A and CFU-S recovery profiles are similar with supra-normal levels on days 12 to 15. There is an additional peak of CFU-A at day 8 that is not reflected in the CFU-S profile, but since the CFU-S are measured at day 12 in the irradiated mice, it is conceivable...
that the CFU-A assay is also detecting more mature day 8 CFU-S after 5-FU treatment. The GM-CFC follow a different pattern with a slight peak on day 8 and a quick return to normal values thereafter. These data are consistent with CFU-A being similar to CFU-S, but do not exclude the possibility that there is some overlap with the GM-CFC compartment. A rigorous determination of f values (seeding with CFU-A being similar to CFU-S, but do not exclude the possibility that there is some overlap with the GM-CFC compartment) on every single time point would be required to further address this issue.

The Effect of CFU-S Regulators on CFU-S and CFU-A

The maintenance of minimal proliferation in normal marrow and the recruitment into rapid proliferation in regenerating marrow are important aspects of stem cell behavior and two regulators affecting the proliferation characteristics of CFU-S (but not GM-CFC or BFU-E) have been described. A factor obtained from normal marrow decreases the proportion of CFU-S in DNA synthesis and a factor obtained from regenerating marrow increases the proportion of normal CFU-S in DNA synthesis. If the CFU-A assay detects a cell similar to that detected by the CFU-S assay, we would expect to obtain similar cell cycle characteristics and responses to these regulatory factors. We found that the proportion of normal marrow CFU-A in DNA synthesis was <10% (Table 2). CFU-S from the same cell sample were similarly quiescent while the GM-CFC were proliferating with 30% in DNA synthesis. The proportion of CFU-A and CFU-S in DNA synthesis was significantly increased by incubation with the CFU-S proliferation stimulator (Table 2). In the converse experiments, CFU-S and CFU-A in phenylhydrazine-treated marrow were rapidly proliferating with >30% in DNA synthesis. Incubation with a CFU-S proliferation inhibitor significantly reduced these proportions and the effect on the proliferation of GM-CFC was not significant. We conclude, therefore, that CFU-A in normal and regenerating marrow do indeed display similar cycling characteristics to the CFU-S and similar responses to the proliferation regulators.

Table 2. The Effect of CFU-S Regulators on the Cycling Status of CFU-A

<table>
<thead>
<tr>
<th>CFU-A (%) in S-phase</th>
<th>Control + Stimulator</th>
<th>Control + Inhibitor</th>
</tr>
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<tbody>
<tr>
<td>CFU-S (%) in S-phase</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>GM-CFC (%) in S-phase</td>
<td>30</td>
<td>31</td>
</tr>
</tbody>
</table>

Results are means of five experiments (SD < 10%) in all cases. Abbreviations: BM, bone marrow; PH, phenylhydrazine bone marrow.

* Significant change from control (P < .001).
† Significant change from control (P < .01).
‡ No significant change from control (P > .05).

Macrosopic Colony Formation With Pure Growth Factors

In our preliminary investigation of the activities present in the conditioned medium from AF1-19T cells we found that the activity synergizing with CSF-1 co-purified with GM-CSF. In addition, we detected GM-CSF transcripts in cellular RNA, but no IL-3 transcripts (data not shown). As the synergizing activity was likely to include GM-CSF, we used recombinant murine GM-CSF9 in combination with L929 CM or pure CSF-1 in the assay (Table 3). These factors acted synergistically to give identical mixed macroscopic colonies and the synergism could be blocked with CSF-1 antiserum, but not by IL-1 antiserum (Table 3). These results show that pure growth factors can be used synergistically to induce primitive multipotential cells to form mixed macroscopic colonies in vitro. Furthermore, since the IL-1 antiserum was present throughout the incubation, we conclude that IL-1 (or hematopoietin-1), if present in AF-19T CM, does not contribute to colony formation in this assay nor is it likely to be induced in an accessory cell in the in vitro assay described here.

DISCUSSION

It is becoming increasingly evident that synergizing growth factors can be used to recruit primitive cells into proliferation and differentiation and that the type of primitive cell recruited from the heterogeneous stem cell compartment may depend on the combination of growth factors used. We describe here an in vitro assay for a primitive cell with similar characteristics to the CFU-S defined by the in vivo assay.

The CFU-A colonies are derived from a cell that has multipotential capacity in vitro and displays self-renewal properties. The cells from developing colonies at day 7 in the assay show some self-renewal capacity, although the conditions we describe may be appropriate for initiating proliferation and differentiation of primitive cells similar to the CFU-S. However, these conditions may not be optimal for self-renewal in vitro. In this context, Koike et al. have shown that low concentrations of IL-3 added to cultures of spleen cells favor development of blast cell colonies with high but variable replating efficiencies. Thus, the cells that form the blast cell colonies may represent a more primitive sub-compartment of the CFU-S with low incidence but high replating efficiency. It would be of interest therefore to
supplement the growth factor cocktail we use with low concentrations of IL-3. Similarly, the HPP-CFC first detected by Bradley and Hodgson,7 subsequently shown to be responsive to IL-1 (hematopoietin-I) and CSF-1 by Stanley et al9 together with the MIX-CFC2 and CFU-GEMM13 also represent sub-compartments of the total primitive cell population. The CFU-A assay we describe here does not require added erythropoietin to demonstrate mixed erythroid colony formation. However, low levels of erythropoietin may be present in the batches of fetal calf serum or horse serum used. It is perhaps significant that a preponderance of more immature erythroid cells is observed in the day 7 to 9 colonies. It is likely therefore that delayed addition of erythropoietin to the cultures at around day 8 may result in emergence of mature erythroid cells in the macroscopic colonies at day 11.

That the combination of growth factors (crude or purified) used here can induce macroscopic colony formation derived from a cell with similar characteristics and incidence to the CFU-S underlines the general usefulness and potential of the assay. The results presented here indicate that the combination of GM-CSF and CSF-1 in these cultures results in the recruitment, proliferation, and differentiation of multipotential stem cells. There are a number of unique applications and superior features of the assay. The macroscopic colony formation affords simple and rapid assessment of primitive colony forming cells whose incidence has relatively little variation in normal bone marrow from various strains of mice. This assessment could be automated using an Image Analysis System.22 The in vitro assay does not require preculture in liquid medium4 nor pretreatment of mice with 5-FU.7,8

In view of the size of the CFU-A colonies and the characteristics of the clonogenic cell, the assay we describe could be useful for the introduction of retrovirus based vectors into stem cells and for the analysis of expression of introduced genes. However, since the assay detects a multipotential cell with a relatively high incidence and characteristics in common with the spleen colony-forming cell a more immediate and unique application of our observations will be the identification and purification of stem cell regulators. These general approaches should facilitate the investigation of the cellular and molecular mechanisms underlying the control of stem cell behavior in normal and neoplastic states. Furthermore, the availability of recombinant human growth factors means that the assay may be used to study the human counterparts of the murine CFU-A. Current experiments of ours suggest that this is now possible.

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The effect of stem cell proliferation regulators demonstrated with an in vitro assay

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