Chapter 23

High Proliferative Potential Colony Forming Cells

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1. Introduction

High proliferative potential colony forming cells (HPP-CFC) in mouse bone marrow (BM) were defined functionally by their ability to form large colonies, greater than 0.5 mm diameter, and containing an average of 5 x 10^4 cells, in low-cell-density nutrient agar cultures after 10–14 d of incubation (*1*).

HPP-CFC were initially detected in bone marrow cells taken from mice after 5-fluorouracil (FU) treatment by virtue of the fact that large colonies developed in culture in response to a combined growth factor stimulus of pregnant mouse uterus extract (PMUE) (2) or CSF-1 from L cell conditioned medium (3) (macrophage colony stimulating factors, M-CSF) plus a source of synergistic factor(s) (SF) (*1,4,5*), but not with either factor alone. Colonies that developed with M-CSF alone were low in numbers, less than 0.5 mm diameter, and contained from 50 to 5 x 10^3 cells with the majority of colonies at the smaller end of the range (*1*). These are desig-

nated as derived from low proliferative potential colony forming cells (LPP-CFC). At 2 d after FU treatment, the LPP-CFC are drastically depleted to less than 0.1% of the numbers present in normal bone marrow whereas the HPP-CFC are 4% of normal. HPP-CFC have been shown to regenerate more rapidly than LPP-CFC, and their regeneration pattern correlates closely with the marrow repopulating ability (MRA) and the platelet repopulating ability (PRA) of post-FU BM, but not with CFU-S developing at 10 or 13 d posttransplantation, and became supranormal in numbers at 8–10 d post-FU before returning to normal at 14–16 d (*4*).

The synergistic factor(s) sources used initially were human, rat, or mouse spleen conditioned media or human placenta (*4,5*). Conditioned medium from human bladder carcinoma cells, 5637, was also shown to be a source of SF in that together with CSF-1 it generated cells from post-FU BM that could be assayed by determination of the increased binding of ^{125}I-CSF-1 and the factor was designated hemopoietin-1 (*6*). With M-CSF, 5637 CM also generates large colonies in agar cultures (*7*). Evidence has recently been produced suggesting that hemopoietin-1 is identical with interleukin-1 (*8*).

Reexamination of the production of large colonies with purified growth factors has shown that a major HPP-CFC population of cells in FU_{2d} BM is optimally stimulated using a combination of the three purified growth factors, CSF-1 plus IL-1 plus IL-3 (*9*). Cells taken later in post-FU regeneration, FU_{8d}BM also respond to other combinations of growth factors, e.g., CSF-1 + IL_3, CSF-1 + IL-1 to produce large colonies, but optimal large colony development still requires the three combined factors (*9*).

HPP-CFC are detectable in normal bone marrow with the growth factor combination of CSF-1 plus IL-1 plus IL-3 at a low incidence: approximately 1 in 400 cells. Other combinations of growth factors can result in large colony formation, for instance, GM-CSF can substitute for M-CSF, and although qualitatively no differences are detectable, quantitatively the colony formation in post-FU bone marrow is less (*9*).

HPP-CFC are among the most primitive hemopoietic cells in mouse bone marrow yet shown to proliferate and differentiate in vitro. The exact relationship of HPP-CFC to CFU-S_{13} is not rigidly defined at present. Using multiparameter cell separative strategies, it is possible to obtain fractions containing up to 30% HPP-CFC and at least 50% CFU-S_{13} (*7*). Cofractionation using these techniques indicates a close relationship, but does not necessarily indicate that they are identical.

2. Materials

1. Agar gels: Stocks of 1 and 0.66% Difco Bacto-agar (*see* Note 1) are made for use over a month. Weigh out the solid agar into a sterile conical screwtopped flask containing a sterile magnetic stirrer bar. Add the requisite amount of sterile distilled water (DW). Stir and heat until the agar is melted and just commences to boil. Cool at room temperature. Repeat melting and boiling. Store at room termperature. It is *important* to make sure the caps are loose during melting and boiling to prevent shattering of the flasks.
2. Culture medium: Double strength medium is made from powdered media. We use alpha-modification of Eagle's MEM (*see* Appendix), but any basic medium that can be made to double strength may be used. Alpha medium has consistently given better results than most other media. For simplicity of daily use involving large amounts of medium, we prepare a more concentrated basic stock, with vitamin supplementation, for freezing and make up to the double-strength medium each day as follows:
   a. Concentrated Stock
      Use alpha powder without nucleosides for 10 L of medium (*see* Note 2). Stir in approximately 1500 mL sterile DW 4–6 h. Add 100 mL Eagle's MEM Vitamins (x100) and 10 mL phenol red (1% aqueous). Determine osmolality and dilute to 1300 mosmol (*see* Note 2), and gas with CO_2. Sterile filter (0.22 µm filters) and store frozen at –20°C.
   b. Double Strength Medium for Plating

Concentrated stock	32 mL
Sterile glutamine (200 m*M*)	2 mL
Sterile serum (*see* Note 3)	40 mL
Sterile sodium bicarbonate (5.6%)	8 mL
Gentamycin (*see* Note 4)	8000 U
Sterile DW	18 mL

      This is 100 mL of double strength medium ready for use (*see* Note 5).
3. Balanced Salt Solution: Hepes buffer (1.5*M*): 35.75 g Hepes (acid salt), 0.2 mL phenol red (1%, w/v). Dissolve in approximately 60 mL distilled water and titrate to pH 7.2 with NaOH. Make up to 100 mL and filter through a 0.22 µm filter and store at 2–8°C.

4. Concentrated Stock Balanced Salt Solution: This is prepared as a x10 concentrated stock solution that is filtered through a 0.22 µm filter and stored at 2–8°C. 80 g/L NaCl, 4 g/L KCl, 10 g/L glucose, 20 mL/L of a 1% (w/v) solution of phenol red, 2 x 10^5 U/L gentamycin.
5. Isotonic Balanced Salt Solution (BSS): 10 mL Hepes buffer (1.5*M* and 5 mL $NaHCO_3$ (5.6%, w/v) is added to 100 mL BSS (x 10) and distilled water is added to bring the volume to 1 L. The solution is filtered through a 0.22 µm filter and stored in aliquots of 100 mL at 2–8°C.
6. Growth Factors: These are prepared at suitable concentrations (*see* text) in single strength medium.
7. Incubation Boxes: Since the dishes should be incubated at 7% oxygen gas phase, we routinely use 5-L capacity polystyrene plastic boxes (*see* Note 6: Stewart Plastics, Purley Way, Croydon CR9 4HS, UK, Cat. No. 212 into which perforated stainless steel trays are placed). The tray is 2 cm above the bottom of the box. Two diagonally opposite holes (18 gage needle size) are drilled in the lids of the boxes.
8. Gas Mixture: Plastic boxes are gassed with a gas mixture of 5% oxygen, 10% carbon dioxide, and 85% nitrogen, which is ordered from regular suppliers (*see* Note 12).

3. Methods

Plating of bone marrow cells in nutrient agar can be made using either a double or a single layer system. We routinely use a double-layer system since colony formation is better than in the single layer system.

3.1. Removal of Marrow

1. Groups of at least five mice are used to provide femurs. The femoral shafts are stripped of all tissue and are flushed from one end and then the other with chilled BSS containing 2% newborn calf serum using a 1 mL syringe fitted with a 23–gage needle.
2. Marrow cells are collected at a concentration of one femur equivalent (2.5 x 10^7 cells)/mL BSS and kept on ice until used.

3.2. Plating Procedure (Double Layer)

1. Sterile growth factors to give the desired final concentrations are added directly to the non-tissue-culture-treated 35-m*M* Petri dishes (Note 7). Routinely the triple growth factor combination is used at the

following doses: CSF-1 1 x 10^3 U, IL-1α 1 x 10^4 U (conversion assay units), IL-3 25 U per dish, but all growth factor preparations should be tested for the concentrations necessary to achieve optimal colony formation.

2. Melt the 1% agar in a conical flask to just boiling and put in a 37°C bath to cool.
3. Calculate the amount of double strength medium to be used for the number of dishes to be plated and allow extra to ensure that the medium-agar mixture will be adequate in volume. Place in a conical screw topped flask and warm in the 37°C bath.
4. When the agar is cooled to 37°C (test it by agitating it in the flask and feeling the temperature), add an equal volume of it to the double strength medium mix thoroughly (either by pipet or with a sterile magnetic stirrer bar previously placed in the flask) and dispense 1 mL to each of the dishes, shaking the dishes from side to side to ensure complete coverage of the dish and mixing with growth factors (Note 8). This basal layer should gel within a few minutes at room temperature (20°C).
5. For the overlay (containing the bone marrow cells), melt and just boil the 0.66% agar and place in the bath to cool (Note 9). Add the required number of bone marrow cells to prewarmed medium (double strength) and immediately add an equal volume of agar. After mixing thoroughly, dispense 0.5 mL aliquots to the dishes; no shaking is required. After a few min the upper layer should be gelled and the dishes can be incubated (*see* Note 10). For normal bone marrow we routinely use 2.5 x 10^3 cells per dish: for FU_{2d}, 2 x 10^4; for FU_g, 1 x 10^3. For fractionated enriched bone marrow populations, the numbers are reduced appropriately (Note 11).
6. 50 mL of sterile DW is placed under the stainless steel tray of the plastic box to provide humidity during the incubation. Stack the dishes in the box. Each box can take up to 150 dishes.
7. The box lids are then taped on with three layers of tape which is left taut and wound around the lid-bottom box junction. No wrinkles should be evident and the taping should be finished by smoothing it down over the junction.
8. The boxes are then gassed at the rate of 2.5 L/min for 30 min to give a final oxygen concentration of 7%, the gassing holes are sealed with three layers of tape, and the box is placed in the 37°C room or incubator (Note 12).

9. At 14 d of incubation, the colony numbers are counted (Notes 13–15) using a dissecting microscope at 20x magnification with a calibrated grid in one eyepiece to measure colony diameters. Normally the colonies originating from HPP-CFC are clearly visible without magnification but are checked to observe that they are tightly packed with cells and to count any smaller colonies (Note 14).

4. Notes

1. Various agarose preparations may be used instead of agar, provided that it is one gelling adequately at room temperature, but colony formation with myeloid cells is lower than in agar. Likewise autoclaving of agar reduces colony formation and should be avoided (*10*).
2. Various other media have been used. It is important to measure the osmolality of media and sera to be used in order to ensure uniformity of culture conditions. On testing a range of media, we have found a final medium osmolatity of 280–300 mosmol to be optimal for mouse bone marrow colony formation.
3. Batches of sera must be tested to choose a pool suitable for the next 6 mo to 1 yr work. We find some batches of newborn calf serum to be better than fetal calf serum.
4. Gentamycin has been used routinely for several years to replace penicillin and streptomycin because of its spectrum and stability.
5. The bicarbonate concentration used gives strong buffering with the 10% carbon dioxide in the gas phase.
6. The plastic boxes are washed and dried before using and between incubations and have several advantages over normal incubators:
   a. Each experiment can remain undisturbed over the incubation period.
   b. Contamination during incubation is not a problem.
   c. They are cheap, and, if a 37°C room, or nonhumidified incubator, is available the number of workers who can carry out clonogenic experiments is greatly increased for little change in cost.
   d. They can be used to test numerous gas concentrations for various cell and culture types.
7. Growth factor preparations should not be added to the underlay at more than 0.3 mL/dish and preferably left to 0.15 mL maximum making 10% of the total double layer system in order to avoid the gels losing their firmness.

8. For plating large numbers of dishes, e.g., above 100 dishes, Cornwall continuous pipets are used for both underlays and overlays with aluminum foil covered or cotton plugged flasks replacing the screw topped flasks.
9. For the single layer system the 0.66% agar is melted and placed in the 37°C bath. The volume of double strength medium is placed in another flask and warmed, the requisite numbers of cells are added, and the equal volume of agar added immediately mixed and plated.
10. The most common failure of cultures arises from using agar that is too hot or over-heating pipets during routine flaming of them. On the other hand, care must be exercised to ensure that the agar-media mixture does not gel before dispensing into the dishes.
11. It has been observed that colony formation with fractionated enriched populations of bone marrow is usually better than with unfractionated marrow (7).
12. The gassing of the incubation boxes is calculated to pass 75 L of gas through the 5-L vol boxes. A brief passage of gas will not suffice to achieve the final 7% oxygen used nor an adequate CO_2 concentration.
13. One of the most important aspects of optimal colony formation in vitro is to ensure that low cell densities are used:
    a. to prevent possible secondary effects occurring such as stimulation of accessory cell production of growth factors,
    b. to prevent the smaller colony formation that can occur when too many colonies develop in the dish.

    When the incidence of cells present at low frequency in the population is being measured, the number of replicate dishes must be increased rather than the cell density per dish. In practice, 20–50 colonies/dish is an optimum to achieve, and especially with an enriched population, dishes should be set up at different cell densities.
14. Colonies from different bone marrow populations may develop at different rates, e.g., using FU_{2d} BM cell cell's clones are just starting to develop at 6 d of incubation and achieve their large colony formation rapidly over the next 4–8 d. FU_{8d} BM develop more rapidly and are often large in diameter at 8–10 d of incubation.
15. HPP-CFC have been detected in all strains tested, e.g. BALB/C, C57-Bl6, AKR, C_3H/HeJ, CBA (C57Bl x BALB/C) -F1, (C57Bl x DBA/2)-F1.
16. Since colony formation takes place over a lengthy incubation period (10–14 d), the HPP-CFC may develop by sequential action of the growth factors initially placed in the cultures on cells generated with-

in the colonies during colony development. Also, combinations of other growth factors than those discussed here may detect cells with high proliferative potential with their actions being either additive or synergistic.

17. At the present time the size of the colony, and more particularly the number of cells generated per single HPP-CFC, are the criteria for detection of these cell types. The development of large colonies in the primary cultures at 14 d does not exhaust their total proliferative potential since these colonies can be sampled, dispersed into single cells, and replated to give further colony formation, although the secondary colonies are smaller and no longer require all of the three growth factors. For example, large colonies developed from $FU_{2d}BM$ cells with CSF-1 plus IL-3 plus 5637 conditioned medium can be replated with CSF-1 alone to yield ultimately 27 x 10^6 cells/initial HPP-CFC. This may demonstrate local exhaustion of the medium during the primary colony formation and/or local inhibitory effects within the tightly packed colonies.

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