IN VITRO CLONING OF MURINE MEGAKARYOCYTE PROGENITORS (CFU-Meg)

Martin J. Murphy, Jr. and Kiyoyuki Ogata

Hipple Cancer Research Center, 4100 South Kettering Boulevard, Dayton, Ohio 45439

SUMMARY: Convenient and reproducible culture systems for murine megakaryocyte progenitor cells (colony-forming unit-megakaryocytes, CFU-Meg) are described. Mouse bone marrow cells are cultured in fibrin clots supplemented with Iscove's modified Dulbecco's medium and fetal bovine serum or in fibrin clots supplemented with serum-free IMDM, bovine serum albumin, transferrin, cholesterol, and L-α-phosphatidylcholine. In the presence of murine interleukin-3 or pokeweed mitogen-stimulated murine spleen cell-conditioned medium, these cultures support CFU-Meg colony formation effectively. The cultivation and counting of colonies in these culture systems is considerably easier when compared with previously reported culture systems.

Key words: fibrin clot; megakaryocyte progenitor cell; colony-forming unit-megakaryocyte (CFU-Meg); serum-free media.

I. INTRODUCTION

Platelets, the cytoplasmic fragments of megakaryocytes, play a key role in hemostasis and in the development of thrombosis and atherosclerosis. This essential blood cell component is derived from pluripotent hematopoietic stem cells through a complex process including commitment of pluripotent stem cells to committed megakaryocyte precursors, proliferation of this cell compartment, differentiation and maturation of these precursors to fully maturated megakaryocytes, and platelet release (2,4,8). This process is regulated by a network of cytokines and cell interactions (1,2,8,11); however, the exact control mechanism of the process is still unclear. Also, there is a constellation of platelet disorders in which the control mechanism(s) of megakaryocytogenesis and platelet production seems to be deranged (11).

Clonogenic culture systems for megakaryocyte progenitor cells (colony-forming unit-megakaryocyte-CFU-Meg) are powerful tools with which to elucidate the mechanisms of megakaryocytogenesis and platelet production in normal and abnormal states. Since the establishment of the clonogenic culture system for murine CFU-Meg (5), several modified systems have been reported in attempts to improve the sensitivity and simplicity of the assay (3,6,9). Recently, in an attempt to eliminate the ill-defined effects of serum and plasma in the assay, they have been successfully replaced with defined constituents (12,13).

We describe two culture systems developed in our laboratory: serum-containing and serum-free fibrin clot culture assays for murine CFU-Meg (3,10). Using these improved and simplified assays, the cultivation and counting of colonies is considerably easier and more reproducible than previously reported systems.

II. MATERIALS

A. Equipment

CO2 incubator, model 3326, Forma Scientific®
Centrifuge, model TJ-6, Beckman®
Laminar flow hood, Sterilgard model SG-600, Baker Company®

B. Chemicals

Iscove's modified Dulbecco's medium (IMDM), no. 1 7633, Sigma®
Fetal bovine serum (FBS), no. A-1115-L, HyClone®
Fibrinogen, no. F 4753®
Thrombin, no. T 9000 and T 7513®
Bovine serum albumin (BSA) Fraction V, no. 12659, Behring Diagnostics®
Transferrin, no. 652-202, Boehringer Mannheim®
Cholesterol, no. C 8254®
L-α-Phosphatidylcholine, no. P 6263®
Pokeweed mitogen (PWM), no. 670-5360, GIBCO®
Recombinant murine interleukin 3 (rIL-3), no. MIL-3, Genzyme®
Penicillin G (sodium salt), no. 860-1830®
Streptomycin sulfate, no. 860-1860®
AG-501 XB (D) resin, no. 143-6425, Bio-Rad®
Sodium bicarbonate, no. 895-1810®
Sodium phosphate monobasic (anhydrous), no. S-0751®
Sodium phosphate dibasic (anhydrous), no. S-0876®
Glutaraldehyde, no. G-6257®
Acetylthiocholine iodide, no. A-5751®
Citric acid (trisodium salt), no. C 7254®
Cupric sulfate (anhydrous), no. C-495, Fisher®
Potassium ferricyanide, no. P-232®
C. Supplies
Vacuum filter system, no. 25943 (0.45 μm), Corning
Syringe filter, no. 190-2045 (0.45 μm), Nalgene
Culture tube, polypropylene, no. 2059 and 2063, Becton
Dickinson
B6D2F, male mice (8-12-wk old), Harlan-Sprague
Dawley
Tissue culture dish, no. 5221-R Lux, Nune
Tissue culture flask, no. 3023

III. PROCEDURE
A. Preparation of solutions
1. Iscove's modified Dulbecco's medium
   a. Dissolve the following in double glass-distilled water (DGDW) and make up to 1 liter
      IMDM 17.7 g
      Penicillin G 30 mg
      Streptomycin sulfate 50 mg
      Sodium bicarbonate 3.024 g
   b. Sterilize using vacuum filter system
   c. Store at 4 °C
2. Double-strength IMDM
   a. Dissolve the following in DGDW; make up to 500 ml
      IMDM 17.7 g
      Penicillin G 30 mg
      Streptomycin sulfate 50 mg
      Sodium bicarbonate 1.512 g
   b. Sterilize using vacuum filter system
   c. Store at 4 °C
3. Fetal bovine serum
   a. Incubate at 56 °C for 30 min
   b. Store in aliquots of 20-100 ml at --20 °C
   Note: To obtain a suitable batch of FBS, several batches should be tested to establish their capacity to support murine CFU-Meg growth.
4. Phosphate buffer
   a. 0.1 M sodium phosphate monobasic
      Dissolve 12 g of sodium phosphate monobasic in DGDW and make up to 1 liter
   b. 0.1 M sodium phosphate dibasic
      Dissolve 14.2 g of sodium phosphate dibasic in DGDW and make up to 1 liter
   c. 0.1 M sodium phosphate buffer, pH 6.0 and 7.1
      Titrate the desired pH solutions of 0.1 M sodium phosphate buffer by mixing 0.1 M sodium phosphate monobasic and dibasic solutions using a pH meter
   d. Store at room temperature
5. Fibrinogen solution
   a. On the day of marrow harvest and culture, dissolve fibrinogen in 0.1 M sodium phosphate buffer (pH 7.1) at a concentration of 2.5 mg/ml
   b. Sterilize using syringe filters
6. Thrombin solution
   a. Dilute thrombin solution with IMDM to yield a concentration of 5 U/ml
   b. Sterilize using syringe filters
   c. Store in aliquots at --20 °C
7. 7.5% Sodium bicarbonate solution
   a. Dissolve 7.5 g of sodium bicarbonate in DGDW and make up to 100 ml
   b. Sterilize using syringe filters
   c. Store at 4 °C
8. Bovine serum albumin solution
   a. Overlay 10 g of BSA powder on 40 ml of DGDW and allow to dissolve without any manipulation at 4 °C overnight
   b. Add 1 g of AG-501 XB (D) resin for 1 h and mix occasionally. Repeat two more times
   c. Centrifuge for 5 min at 400 g
   d. Harvest supernatant and dilute two-fold with double-strength IMDM
   e. Adjust to pH 7.1 with 7.5% sodium bicarbonate solution
   f. Sterilize using syringe filters
   g. Store in aliquots at --20 °C
   h. Adjust the pH again before use, if necessary
9. Transferrin solution
   a. Using aseptic techniques, dilute transferrin solution with IMDM to yield a concentration of 6 mg/ml
   b. Store in aliquots at --20 °C
10. Cholesterol and L-a-phosphatidylcholine suspension
    a. Dissolve 100 mg of cholesterol in 1 ml of L-a-phosphatidylcholine-chloroform solution (100 mg/ml)
    b. Place each 50 μl of this solution in a culture tube and evaporate chloroform by vacuum
    c. Store dark at --20 °C
    d. When ready for use, add 5 ml of IMDM to the tube and sonicate
    e. Sterilize using a syringe filter
11. Glutaraldehyde solution
    a. Dilute glutaraldehyde solution with DGDW to yield a concentration of 5%
    b. Store at 4 °C
12. Citric acid solution
    a. Dissolve 29.41 g of citric acid in DGDW and make up to 1 liter
    b. Store at 4 °C
13. Cupric sulfate solution
    a. Dissolve 0.48 g of cupric sulfate in DGDW and make up to 100 ml (i.e. a 5 mM solution)
    b. Store at 4 °C
14. Potassium ferricyanide solution
    a. Dissolve 0.165 g of potassium ferricyanide in DGDW and make up to 100 ml (i.e. a 5 mM solution)
    b. Store at 4 °C
15. Staining solution for acetylcholinesterase
    a. Just before use, dissolve 100 mg of acetylthiocholine iodide in 150 ml of 0.1 M sodium phosphate buffer, pH 6.0
    b. Add 10 ml of citric acid, 20 ml of cupric sulfate, and 20 ml of potassium ferricyanide solutions in that order
B. Preparation of pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM)