Regulation of Megakaryopoiesis in Long-Term Murine Bone Marrow Cultures

By Neil Williams, Heather Jackson, A. P. C. Sheridan, Martin J. Murphy, Jr., A. Elste, and Malcolm A. S. Moore

Megakaryocytes and their precursor cells were sustained in mouse bone marrow suspension cultures for over 4–6 wk. Megakaryocyte precursor cells were detected by their capacity to form colonies of megakaryocytes in semisolid agar cultures. Colony formation was dependent on the presence of medium conditioned by a myelomonocytic leukemic cell line (WEHI-3CM). Megakaryocytes from the liquid and semisolid cultures were identified by cytoplasmic acetylcholine esterase and by ultrastructural analysis. The suspension medium from the bone marrow liquid cultures which sustained megakaryopoiesis was not directly active in stimulating megakaryocyte colony formation in the semisolid agar cultures, but potentiated the number of colonies detected when WEHI-3CM was present. Bone marrow-conditioned medium increased the sensitivity of megakaryocyte progenitor cells to the stimulus in WEHI-3CM. Addition of the activities present in the two sources produced a quantitative assay for the detection of mouse megakaryocyte progenitor cells. These studies showed: (1) that no inductive regulator of in vitro clones of megakaryocytes was present in the supernatants from the long-term marrow cultures and, (2) that at least two factors were necessary for the induction of megakaryocyte progenitors to proliferate and differentiate in semisolid cultures in vitro.

Megalakaryocytes and their precursor cells are normally present in low incidences in bone marrow, and little is known of the development of ploidy from unrecognizable mononuclear cells and of the maturation events leading to platelet formation. Recently a long-term culture system of mouse bone marrow cells has been developed in which granulopoiesis is sustained over many weeks from a self-renewing pluripotent stem cell compartment. Although the stem cells remain truly pluripotent when injected into lethally irradiated recipients, their commitment in vitro has hitherto been restricted to the production of neutrophils and macrophages. These cultured cells are identical to freshly isolated granulocytic cells in their biologic and biophysical properties. This selectivity apparently results from the inability to induce and sustain other cell lineages.

In this communication, we have shown that mouse megakaryocytes and their precursor cells are produced in these cultures over several weeks. Progenitor cells of all hemopoietic cell lineages, including megakaryocytes, have been detected in semisolid or plasma clot cultures of murine marrow. The role of obligatory stimulators in the induction and regulation of precursor cells of granulocytes and macrophages, erythrocytes, and lymphocytes has also been...
studied using the same assay systems. The semisolid agar culture procedure is used in this study to measure the numbers of megakaryocyte progenitor cells and to assess the role of moieties regulating megakaryopoiesis in long-term bone marrow cultures.

**MATERIALS AND METHODS**

**Cells**

Bone marrow cells were obtained from (DBA/2 x C57BL/6)F1 mice (Cumberland Farms) by flushing the marrow cavity with cold phosphate-buffered saline containing 5% fetal calf serum (FCS). A single cell suspension was obtained by repeated pipetting, and viable cell counts were obtained on the basis of exclusion of trypan blue.

**Establishing Long-Term Bone Marrow Cultures**

The original method was modified as described elsewhere. Briefly, bone marrow cells from a single femur were syringed directly into 25 sq cm plastic flasks (Corning Plastics). No attempt was made to break up marrow aggregates. The cells were incubated at 33°C in 10 ml of Fisher’s medium (Gibco) containing 20% horse serum (Flow). After either 3 or 4 days, half the medium containing half of the suspension cells was removed and the cultures were fed with an equal volume of fresh medium. After 7 days, all the nonadherent cells were removed and 3-5 x 10⁶ freshly isolated bone marrow cells were added in new medium. Each week half the cells were harvested from 3-5 culture flasks, pooled, and assayed for pluripotent hemopoietic stem cells (CFU-S) and committed progenitor cells of both granulocytes-macrophages (CFU-C) and megakaryocytes (CFU-M). The differential morphology of the cells was assessed using standard Giemsa stain.

**Cloning of Pluripotent Hemopoietic Stem Cells In Vivo**

Spleen colony-forming units (CFU-S) were quantitated using the technique of Till and McCulloch. Pooled suspension cells or normal bone marrow cells (25,000) were injected into groups of six lethally irradiated (850 rads) B6D2F1 recipient mice. After 9 days, the spleens were removed, fixed in Bouin’s solution, and scored for macroscopic spleen colonies.

**Cloning of Megakaryocyte and Granulocyte-Macrophage Progenitor Cells In Vitro**

CFU-M were cloned in semisolid agar culture using a modification of the technique of Metcalf et al. Cells from femoral bone marrow were cultured at concentrations of 10⁴ to 2 x 10⁵ cells/ml in 35-mm Petri dishes (Falcon 3001) in McCoy’s 5A modified medium containing 15% FCS and 0.3% Bactoagar supplemented with essential and nonessential amino acids, 200 mM glutamine, asparagine, sodium pyruvate, and 10⁻⁴ M 2-mercaptoethanol. Megakaryocyte-stimulating activity was obtained by adding 100 µl of a 10-fold concentration of serum-free conditioned medium (approximately 700 µg protein/ml) from cultures of the WEHI-3 murine myelomonocytic leukemic cell line.

The BALB/c myelomonocytic leukemic cell line has been adapted to growth in suspension culture. The cells were propagated in 75 sq cm plastic flasks in McCoy’s 5A medium with 15% FCS and 5 x 10⁻⁵ M 2-mercaptoethanol. Continuously growing cultures were cycled for 1 wk in serum-free medium (cell density 1 x 10⁶ cells/ml); the serum medium was harvested, centrifuged at 16,000 g for 30 min, and then dialyzed against three changes of distilled deionized water at 4°C for 3 days. The conditioned medium was again centrifuged and concentrated 10-fold using Amicon ultrafiltration with a PM 10 membrane. Cultures were scored at x40 magnification for CFU-M after 7 days of incubation at 37°C in a humidified incubator. WEHI-3-conditioned medium (WEHI-3CM) was also a source of activities required for the cloning and differentiation of CFU-C. After 7 days of culture, the WEHI-3CM stimulated approximately 20% granulocyte, 30% mixed granulocyte and macrophage, and 50% macrophage CFU-C.

Colonies of megakaryocytes were readily identified by the size of the majority of colony cells. They were generally irregular in shape and were markedly larger (50 μm) than the cells which
Fig. 1. Macroscopic morphology of colonies stimulated by WEHI-3CM. Colonies were photographed 7 days after culture. (A) Megakaryocyte colony. (B) Granulocyte and granulocyte-macrophage colonies. (C) Macrophage colony. ×40.
comprised either granulocyte or macrophage colonies. Each megakaryocyte colony was generally made up of fewer cells (<50 cells) than the typical granulocyte or macrophage colony (Fig. 1). Cytological identification of both large and small megakaryocytes was obtained on isolated colony cells by staining for the presence of acetylcholine esterase.8,15

**Electron Microscopic Analysis**

Megakaryocytes clonally derived from CFU-M were examined ultrastructurally using a modification of the semisolid agar culture technique.16 Bone marrow cells from 1-3-wk suspension cultures were plated in 0.3% agar over 1 ml of a 0.5% agar layer containing 10% WEHI-3CM in 35-mm plastic Petri dishes (Falcon). These cultures contained 100 μl of WEHI-3CM and 200 μl of the long-term bone marrow-conditioned medium (bmCM). After 7-10 days of incubation, the entire contents of each Petri dish were fixed in 2% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4). The dehydration and embedment were completed with the colonies remaining in place within the 0.3% agar, forming an Epon disk. Selected colonies of cells were cut from the disk with a jeweler’s saw, thin-sectioned with a diamond knife, and stained with uranylacetate and Reynolds’ lead citrate stain.17 A Siemens IA electron microscope was used throughout.

**RESULTS**

**Dose-Response Relationship Between Myeloid Progenitor Cells and Stimuli Present in WEHI-3CM**

Approximately 250 CFU-C and 7.5 CFU-M/10^5 bone marrow cells could be stimulated with the concentrations of WEHI-3CM concentrated 10-fold as in Materials and Methods. The dose-response of CFU-C and CFU-M to the stimuli is given in Fig. 2 using WEHI-3CM that had been concentrated 80-fold. The activities stimulating CFU-C and CFU-M titrated over a 100-fold dilution. The plateau levels obtained for CFU-M may be misleading as CFU-C formation was inhibited at the highest concentration of WEHI-3CM. For routine analysis 100 μl of 10-fold concentrated WEHI-3CM was used as a source of stimulus.

**Continued Presence of Megakaryocytes and Their Progenitor Cells**

The persistence of CFU-S in the long-term cultures and the continuous production of CFU-C over an 8-wk period was confirmed. A representative culture is characterized in Table 1. The selective loss of erythroid and lymphoid elements in the cultures resulted in a fivefold enrichment of CFU-C so that the latter comprised approximately 1.5% of the cells recovered at each weekly de-
Regulation of Megakaryopoiesis in Long-Term Cultures

### Table 1. Continued Megakaryopoiesis in Long-Term Cultures

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Cell Count (x 10(^{-5}))</th>
<th>CFU-S</th>
<th>CFU-C per Culture</th>
<th>CFU-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated input</td>
<td>45.0</td>
<td>1125</td>
<td>11,000</td>
<td>900</td>
</tr>
<tr>
<td>Weeks cultured</td>
<td>1</td>
<td>14.0</td>
<td>395</td>
<td>14,000</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10.0</td>
<td>468</td>
<td>10,800</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>15.0</td>
<td>396</td>
<td>27,300</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10.0</td>
<td>208</td>
<td>17,480</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Cell Count (x 10(^{-5}))</th>
<th>CFU-S</th>
<th>CFU-C per Culture</th>
<th>CFU-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated input</td>
<td>40.0</td>
<td>1000</td>
<td>10,000</td>
<td>800</td>
</tr>
<tr>
<td>Weeks cultured</td>
<td>1</td>
<td>11.0</td>
<td>NT</td>
<td>14,800</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13.0</td>
<td>NT</td>
<td>25,500</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.5</td>
<td>NT</td>
<td>12,000</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.0</td>
<td>NT</td>
<td>11,000</td>
</tr>
</tbody>
</table>

*The estimated input was calculated from the cell count when a specified number of bone marrow cells were added to the preformed adherent layer.

*NT: not tested.*

...population. Megakaryocyte production persisted for up to 6 wk. Greater than 1% of all cultured cells were identified as megakaryocytes by acetylcholine esterase staining. Progenitor cells for megakaryopoiesis were continuously produced over the same period of time (Table 1).

Megakaryocytes were analyzed for ultrastructural components indicating megakaryocyte maturation and platelet release. Cells were obtained from colonies derived from megakaryocyte progenitor cells generated in the liquid cultures.

Figure 3 shows representative megakaryocytes and platelets after 10 days of culture in semisolid agar. Megakaryocytes were often replete with surface-connecting demarcation membranes, sometimes showing compartmentalization of the cytoplasm into a focus of demarcation membranes and foci of granules. The cells' multilobulated nuclei frequently displayed prominent nucleoli. The cytoplasmic constituent common to both megakaryocytes and the platelets they produce was the dense \(\alpha\) granules, each with well-formed nucleoid elements.

### Regulators of Megakaryopoiesis in Long-Term Marrow Cultures

Since megakaryopoiesis is thought to be induced by a humoral regulator, thrombopoietin, evidence was sought for a stimulator in the supernatant of the long-term bmCM. Medium was taken from the cultures between weeks 1 and 9 and tested for its ability to stimulate directly megakaryocyte differentiation of progenitor cells derived either from freshly isolated bone marrow or from 1–3-wk-old continuous marrow cultures. No colonies developed using the bmCM alone as a source of stimulator. The megakaryocyte progenitors from both sources did, however, respond to the stimulus present in the WEHI-3CM.

The bmCM may have contained nonspecific inhibitors which prevented megakaryocyte development in the agar cultures. Mixing experiments were performed in which bmCM was added together with WEHI-3CM to the cultures. While no change in the incidence of the CFU-C stimulated by the WEHI-3CM was observed, the number of CFU-M per plate was enhanced (Table 2).
Fig. 3. Ultrastructure analysis of megakaryocytes from day 10 cultures. (A) Megakaryocytes and platelets. × 7000. (B) Field of shed platelets and associated megakaryocyte. × 2400. (C) Platelets containing α granules. × 11,000.
Fig. 4. Effect of increasing concentrations of the supernatants from the long-term bone marrow liquid cultures (bone marrow-conditioned medium) on the incidence of CFU-M (○) and CFU-C (■) cloned in vitro. Bone marrow-conditioned medium alone (■) did not stimulate the growth of CFU-M.

Table 2. Incidence of CFU-M in Primary and Cultured Marrow Cells

<table>
<thead>
<tr>
<th>Source of Responsive Cells</th>
<th>CFU-M/10⁵ Cells Plated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WEHI-3CM* (± SD)</td>
</tr>
<tr>
<td>Primary marrow cells</td>
<td>7.5 ± 4.4 (16)†</td>
</tr>
<tr>
<td>Cultured bone marrow cells</td>
<td>19.8 ± 8.2 (17)</td>
</tr>
</tbody>
</table>

*WEHI-3CM: conditioned medium of myelomonocytic cell line WEHI-3.14
†bmCM: bone marrow-conditioned medium taken from long-term cultures during weeks 3–9.
††Number of observations is in parenthesis.
§Cultured bone marrow cells taken from cells harvested from liquid cultures during weeks 1–4.

The potentiating effect was maximized by adding 200 μl of bmCM to the plate (Fig. 4). The titration of the bmCM was determined by altering the proportion of conditioned medium to fresh medium, while keeping the final volume in the plate constant. Inhibition was seen at the highest concentration of bmCM used, possibly due to nutrient deficiency in the culture plates. Since the bone marrow cells in the plate may condition the cultures over the 7-day incubation period, the number of cells plated was minimized (50,000 cells/plate).

Influence of bmCM on CFU-M formation

The relationship between the number of cells plated and the number of megakaryocyte progenitor cells was only quasi-linear using the cloning techniques previously described.7,8 Deviation from linearity was found at high cell concentrations, implying that bone marrow cells themselves were, in fact, potentiating colony incidence. These assays were not quantitative either; i.e., the plot of the relationship between colony number and number of cells plated did not pass through zero. When CFU-M were scored from cultures stimulated with WEHI-3CM, similar results to those previously published7,8 were obtained (Fig. 5). Although the bmCM did not induce colony formation itself, the addition of 200 μl of the medium to these cultures together with 100 μl WEHI-3CM potentiated colony incidence, and a quantitative relationship was obtained between the number of CFU-M cloned and the number of cells present in the plate (Fig. 5). The bmCM was used at plateau levels of activity (20%).

The role of two factors in megakaryocyte colony formation was studied by determining the responsiveness of CFU-M to WEHI-3CM alone and to WEHI-3CM plus optimal levels of bmCM. A typical dose–response curve is shown in
Fig. 6. Megakaryocyte progenitor cells had little responsiveness to WEHI-3CM alone. A 5-8-fold increase in sensitivity to the same stimulator, however, was observed in the presence of both WEHI-3CM and bmCM. The bmCM was inactive alone in inducing CFU-M formation. Since WEHI-3CM also contained activities required for stimulating colony formation from granulocyte-macrophage precursor cells, the CFU-C were scored from the same plates as a specificity control. The responsiveness of CFU-C to WEHI-3CM was not found to be different in the presence of bmCM.

DISCUSSION

Considerable evidence has accumulated to indicate that a humoral factor, thrombopoietin, is responsible for controlling platelet production in vivo. Early studies have indicated that transfer of serum from thrombocytopenic animals to normal recipients results in increased platelet counts. More sensitive in vivo assays have subsequently been developed, utilizing recipient mice in rebound thrombocytosis. Thrombopoietic activity in test preparations injected into such mice can be quantitated by measuring the incorporation of $^{35}$S into platelets.

Recently, in vitro clonegenic assays have been developed which allow detection of thrombopoietic activities. Stimuli have been derived from the conditioned media from PHA-stimulated spleen cells, L cells, and from enriched erythropoietin preparations. In these studies mouse megakaryocyte colony...
formation was dependent on a stimulator present in the conditioned medium from a myelomonocytic leukemic cell line (WEHI-3) which had been concentrated after exhaustive dialysis. The assay detected similar incidences of megakaryocyte colonies to those reported by others,7,8 but was not quantitative, and the relationship between the number of cells plated and the number of CFU-M obtained was only quasi-linear. The addition of bmCM together with WEHI-3CM, however, potentiated the number of CFU-M and allowed a quantitative assay to be developed which detected approximately 25 CFU-M/10⁵ bone marrow cells. Colony formation was not dependent on the presence of 2-mercaptoethanol, unlike B-lymphocyte colony formation.6 This sulfhydryl-containing compound increased the reproducibility of CFU-M detection, as well as the plating efficiency of CFU-C. The effect was optimal at 10⁻⁴ M, a concentration which minimized B-lymphocyte colony formation (Williams, unpublished data).

The site of thrombopoietin-stimulating factor production in the body and the nature of the active cells remains controversial. The kidney, spleen, pituitary, and adrenal glands have all been implicated in thrombopoietin production.20,21 It is very possible that, like the granulocyte-macrophage stimulating factors, thrombopoietin is elaborated by cell types which are common to many tissues. Based on sedimentation rate analysis of spleen cells, Metcalf et al.7 have suggested that mitogen-stimulated small lymphocytes are a source of the megakaryocyte stimulus. Our observations suggest that subpopulations of phagocytic mononuclear cells may play a regulatory role in thrombopoiesis. First, a direct stimulator of megakaryocyte production is obtained from the medium conditioned from an immature monocytic-monoblastic cell line (Fig. 2).14 Second, the potentiating activity could be obtained from the bone marrow culture supernatants from all culture times tested, including cells cultured for 9 wk when 75%-90% of all cells were phagocytic macrophages and the adherent cell layer is diminished (Williams, unpublished data).

Colony size was larger than previously reported7-9 and the difference is thought to be due to the stimulus present in WEHI-3CM. In the presence of the bmCM, the megakaryocyte progenitor cell was at least five times more sensitive to the stimulus in WEHI-3CM (Fig. 6). The bmCM did not influence either the incidence of CFU-C or the responsiveness of CFU-C to granulocyte-macrophage stimulators present in WEHI-3CM, indicating that the active moiety in bmCM may have specificity restricted to the megakaryocyte progenitor cell compartment.

In this study, the production and shedding of platelets from megakaryocytes grown in semisolid agar is reported. The ultrastructural analysis of differentiating megakaryocytes constitutes a subsequent communication.

The continued production of CFU-M in the long-term marrow cultures may result either from differentiation of CFU-S into CFU-M or from replication of CFU-M originally present, or both. Megakaryocytes and their progenitor cells, however, do appear to have a limited capacity to be recruited from the stem cell compartment, compared with the granulocytic cell lineage. Megakaryocyte progenitors are sustained for only a few weeks (Fig. 2) compared with CFU-C. The reason for this is unclear. Certainly there is no lack for differentiation pres-
sure, as the megakaryocyte-enhancing activity is present in the medium many weeks after megakaryopoiesis has ceased. The relatively shorter period of generation of CFU-M relative to CFU-C in continuous marrow cultures may be due to the loss of some general function in the adherent cell populations. Although no qualitative change can be observed by low-power microscopy, the time at which megakaryopoiesis (about 5 wk) decreases is approximately the same time as when the self-renewal capacity of CFU-S and CFU-C starts declining, and the numbers per culture start to diminish. A second explanation for the cessation of megakaryocyte production is that a specific cell is lost within the adherent layer which elaborates the moiety secreted by the WEHI-3 leukemic cell line. This substance is not found in the marrow supernatant and would have to be transferred by cell–cell contact or produced in limiting amounts. Evidence for pinocytotic junctions between marrow cells in the adherent cell layer has been demonstrated, suggesting that cell–cell interactions may be important in the induction mechanism. A third possibility is that the CFU-M actually do have a limited capacity to be recruited from pluripotent stem cells and that the activity present in WEHI-3CM may have no role other than as a clonegenic agent in the culture in vitro; the bmCM, while inactive alone in vitro, may be the true regulator.

While the relationship between megakaryocyte-stimulating activities in vitro and regulators of platelet production in vivo remains undetermined, the availability of these two in vitro culture systems should accelerate considerably our understanding of thrombopoiesis.

REFERENCES


13. Till JE, McCulloch EA: A direct measure-
Regulation of megakaryopoiesis in long-term murine bone marrow cultures

N Williams, H Jackson, AP Sheridan, MJ Jr Murphy, A Elste and MA Moore