Regulation of the Proliferation of Murine Megakaryocyte Progenitor Cells by Cell Cycle

By Neil Williams and Heather Jackson

The extent to which mouse megakaryocyte progenitor cells (colony-forming unit-megakaryocyte, CFU-M) can proliferate in semisolid cultures prior to endomitosis, and conditions that may regulate that differentiation step, were investigated. The proliferative capacity of CFU-M was determined by estimating the number of megakaryocytes per colony. A bimodal distribution was observed (modal values, 10–15 and 25–30 cells/colony), indicating that separate megakaryocyte progenitor cells may be biased in their capacity for proliferation versus endomitosis. Differences were observed in the cell cycle characteristics of CFU-M as determined in vivo and in vitro that suggest that maturation of CFU-M into megakaryocytes may be regulated within the marrow by control of the cell cycle of the megakaryocyte precursor cell.

COLONY ASSAYS in vitro for the detection of progenitor cells of all hemopoietic cell lineages from mouse marrow, including the megakaryocyte progenitor cell (colony-forming unit-megakaryocyte, CFU-M), have been previously described.1–7 Megakaryocytes formed in these cultures are capable of full maturation, since colony megakaryocytes have normal ploidy values4 and shed platelets.6,7 Using appropriate culture conditions, the number of megakaryocyte colonies was found to be directly proportional to the number of bone marrow cells cultured, allowing quantitation of the CFU-M population.7

The clonable megakaryocyte progenitor cell may be the precursor cells of small acetylcholinesterase-positive cells, since the increase in CFU-M precedes the detection of heightened numbers of small acetylcholinesterase-positive cells in experimentally induced thrombocytopenic animals.8,9 This notion is substantiated by analysis of the physical properties of the two cell types. Megakaryocyte colony-forming cells have physical characteristics typical of diploid cells,4,6,8 while small, acetylcholinesterase-containing cells are markedly more dense than the bulk of nucleated cells,10 a property that may reflect increased cytoplasmic maturation and/or nuclear condensation. Thus it is thought that CFU-M represent a population of cells between the pluripotent stem cells and the morphologically identifiable megakaryoblasts.8

The role of obligatory stimulators on the proliferation and regulation of megakaryocyte colony-forming cells has also been investigated using these culture systems.7,8 Two activities may be involved in megakaryocyte formation and maturation. Entities present in mitogen-stimulated spleen cell supernatant,4,5 erythropoietin,6 and the conditioned medium from a myelomonocytic leukemic cell line (WEHI-3)7 all promote directly the growth of megakaryocyte colonies, while factors that are nonstimulatory alone but that potentiate colony

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formation have been detected in thrombopoietin-rich serum from mice treated with antiplatelet antiserum and medium from cultures of bone marrow cells that generate megakaryocyte progenitor cells over several weeks.

In this study, the extent to which megakaryocyte progenitor cells can divide prior to endomitosis and their cell cycle characteristics were analyzed.

**MATERIALS AND METHODS**

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

**Cloning in vitro of megakaryocyte and granulocyte-macrophage progenitor cells.** 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 generally at 75,000 cells/ml in 35-mm Petri dishes (Falcon 3001) in McCoy's modified 5A medium containing 15% FCS and 0.5% Bactoagar and supplemented with 0.8% minimum essential medium (MEM) essential amino acid mixture (x 50), 0.4% MEM nonessential amino acid mixture (x 100), 2 mM glutamine, 16 μg/ml L-asparagine, 8 μg/ml L-serine, 1 mM sodium pyruvate, and 10⁻⁴ M 2-mercaptoethanol. Two activities have been shown to be required for quantitation of CFU-M in mouse marrow. The two entities were obtained for these studies from conditioned media from cultures of the WEHI-3 murine myelomonocytic leukemic cell line WEHI-3 and from long-term cultures of bone marrow cells. CFU-M were grown in cultures containing 50 μl of an eightfold concentration of conditioned medium (approximately 700 μg protein/ml) from cultures of the WEHI-3-conditioned medium (WEHI-3-CM) and 200 μl of bone marrow-conditioned medium (BM-CM).

Cultures were scored at 40x magnification for megakaryocyte colonies (CFU-M) after 7 days incubation at 37°C in a humidified incubator in an atmosphere of 7% CO₂ in air. Megakaryocytes present in the cultures were found to be highly sensitive to the atmosphere after 7 days culture, and disintegration was often observed within 1 hr after removing the cultures from the incubator. Accordingly, plates were removed only immediately prior to scoring.

Colonies of megakaryocytes were readily identifiable by the size of the majority of cells contained in them. WEHI-3-CM also stimulates the growth of macrophage-granulocyte colonies; however, the cells comprising the megakaryocyte colonies were markedly larger than the cells that comprised either granulocyte or macrophage colonies. Each megakaryocyte colony was generally made up of fewer cells (<50 cells) than the average typical granulocyte or macrophage colony (>100 cells). Cytochemical identification of both large and small megakaryocytes was obtained with isolated colony cells by staining for the presence of acetylcholinesterase.

Granulocyte-macrophage colony-forming cells (CFU-c) were grown using the same conditions as for the CFU-M except that BM-CM was not added and generally fewer cells were cultured (30,000 cells/ml). Colonies were scored at 25x magnification under a dissection microscope after 7 days incubation in 7% CO₂ in air at 37°C in a humidified incubator.

**Preparation of WEHI-3-CM.** WEHI-3 has been adapted to growth in suspension culture. The cells were propagated in 75-cm² plastic flasks in McCoy's 5A medium with 2% FCS and 5 x 10⁻⁵ M 2-mercaptoethanol. The medium was harvested every 3 or 4 days, centrifuged at 3000 g for 5 min, and then dialyzed against three changes of distilled deionized water at 4°C over 3 days. The CM was again centrifuged (18,000 g for 30 min at 4°C) and concentrated eightfold using a Pellicon Cassette System (Millipore) with a PTGC 00001 filter (rated 10,000-dalton barrier). WEHI-3-CM is the source of many stimulatory activities, and after culturing bone marrow cells in the presence of WEHI-3-CM for 7 days colonies comprised of granulocytes (8%–34%), mixtures of granulocytes and macrophages (20%–43%), macrophages (29%–66%), and megakaryocytes (1%–5%) were obtained.

**Preparation of BM-CM.** Bone marrow cultures were set up using a modification of the method of Dexter and co-workers as described by Williams et al. Briefly, adherent cell layers were initiated in 25-cm² plastic flasks (Corning) with the bone marrow cells from a single femur suspended in 10 ml Fisher's medium containing 20% horse serum. Half the suspension medium, including nonadherent cells, was removed after either 3 or 4 days and the cultures replenished with
an equivalent volume of fresh medium. After 7 days all the nonadherent cells were removed, and approximately $5 \times 10^6$ freshly isolated cells were added in fresh medium to the adherent cell layer. Half the cells plus medium were harvested each subsequent week for analysis. The BM-CM was separated from the suspension cells by centrifugation. All the media over the time of preparation of the adherent cell layer and the weeks after repopulation were stored frozen at $-20^\circ$C and tested in a single experiment for CFU-M-potentiating activity. Active samples were pooled, divided into small-volume aliquots, and stored at $-20^\circ$C for use.

**Cytocidal experiments.** The proportion of CFU-M in $S$ phase of the cell cycle was determined by incubating bone marrow cells ($3 \times 10^6$ cells/ml) for 30 min at $37^\circ$C in high specific activity tritiated thymidine ($^{3}H$-TdR sp act 20 Ci/mM; New England Nuclear) at a concentration of either 10 or $30 \mu$Ci/ml. The incubations were terminated by adding 5-8 vol ice-cold unlabeled thymidine 200 $\mu$g/ml; the cells were then centrifuged and washed prior to culturing. Similar experiments were made employing hydroxyurea ($2 \times 10^{-3}$ M), which was added to $3 \times 10^6$ bone marrow cells in 1 ml of McCoy's medium and incubated at $37^\circ$C for 60 min. Control cultures without hydroxyurea were also set up. The cultures were terminated by adding 5 ml ice-cold medium, centrifuged, and washed prior to culturing.

For experiments in vivo, hydroxyurea (900 mg/kg) was given intraperitoneally 3 hr before the animals were killed and a suspension of bone marrow cells was prepared for assay. This concentration of the drug has been shown to be a plateau level, and the number of hemopoietic progenitor cells in vivo was reduced by the same extent as other cell cycle-specific agents.$^{18}$

**Lethal irradiation and reconstitution of mice with bone marrow.** Mice received 900 R from a $^{137}$Cs source in a Mark I irradiator (J. L. Shepherd) calibrated at 113 R/min. After irradiation, mice were injected intravenously with approximately $10 \times 10^6$ marrow cells (half a femur) and killed 7 or 10 days later.

**RESULTS**

**Minimum proliferative potential of megakaryocyte progenitor cells.** The extent to which CFU-M can proliferate prior to endomitosis was assessed by counting the number of cells per megakaryocyte clone. The number of large cells in the typically recognizable megakaryocyte colonies was directly counted on the plate at 40× magnification. A modal value of 5–15 cells/colony was obtained (Fig. 1A). Colony size was also assessed by transferring the colonies from culture dishes to glass slides and staining the cells for the presence of intracellular acetylcholinesterase. The colony size distribution thus obtained was bimodal with peak values of 10–15 and 25–30 cells/colony observed (Fig. 1B). Many small mononuclear cells not counted by the direct method were found within the colonies to stain very intensely for acetylcholinesterase, and colonies with as many as 250–300 acetylcholinesterase-positive cells were obtained.

**Cell cycle status of megakaryocyte progenitor cells grown in semisolid agar cultures.** The cell cycle characteristics of megakaryocyte progenitor cells were investigated by incubating matching cultures of freshly isolated bone marrow

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**Fig. 1.** Proliferative capacity of megakaryocyte progenitor cells from freshly isolated bone marrow. Number of cells/colony counted either (A) as large cells directly in the plate (at 40× magnification) or (B) by removing sequential colonies by Pasteur pipette and staining the cells for acetylcholinesterase. Number of colonies counted in each group in parentheses.
75,000 bone marrow cells cultured per plate. Means ± SD from seven plates/group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU-c Reduction (%)</th>
<th>CFU-M Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per Plate</td>
<td>Per Plate</td>
</tr>
<tr>
<td>10 μCi $^3$H-TdR</td>
<td>190 ± 15</td>
<td>9.85 ± 2.1</td>
</tr>
<tr>
<td>30 μCi $^3$H-TdR</td>
<td>127 ± 14</td>
<td>4.9 ± 1.65</td>
</tr>
<tr>
<td>10 μCi $^3$H-TdR + 200 μg TdR</td>
<td>176 ± 16</td>
<td>9.60 ± 1.8</td>
</tr>
</tbody>
</table>

*75,000 B6D2F1 bone marrow cells cultured per plate. Mean ± SD from 20 plates, from one of three similar experiments.

cells, either with high specific activity tritiated thymidine or with unlabeled thymidine, as described in Materials and Methods. The reduction in the number of CFU-M and CFU-c colonies is given in Table 1. The number of CFU-M was reduced by 50%-60% using this procedure. These concentrations of thymidine were not inhibitory, and the effect of the radioisotope was successfully blocked by the simultaneous addition of unlabeled thymidine (Table 1). The number of granulocyte/macrophage colonies present in the same plates was scored, and a reduction of approximately 35% was observed, a value similar to that reported previously. In the course of these experiments it was observed in the control samples that increased frequencies of CFU-M were detected when the bone marrow cells were incubated without thymidine at 37°C, compared to marrow cells maintained for an equal time on ice. For all subsequent experiments, bone marrow cells were routinely incubated at 37°C for 30-60 min in McCoy's medium at a concentration of 3-10 × 10⁶ cells/ml prior to plating. The reduction in the numbers of CFU-M by cycle-specific agents was confirmed by measuring the incidence of CFU-M after incubating bone marrow cells with various concentrations of hydroxyurea (Table 2). The percentage reduction in CFU-M after incubation of bone marrow cells at 37°C for 1 hr with hydroxyurea was essentially the same as with high specific activity tritiated thymidine (Table 1).

Effect of hydroxyurea in vivo on CFU-M and CFU-c levels in the marrow of normal and reconstituted lethally irradiated mice. The number of megakaryocyte progenitor cells per femur in mice treated with 900 mg/kg hydroxyurea was compared with the number of CFU-M in the marrow of untreated mice. The results of six experiments are given in Table 3. It is evident that the CFU-M were less sensitive to the hydroxyurea than were the CFU-c. Generally, fewer

Table 2. Sensitivity of CFU-M In Vitro to Pretreatment With Hydroxyurea

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU-M</th>
<th>CFU-M Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per Plate</td>
<td>Reduction (%)</td>
</tr>
<tr>
<td>$10^{-3}$ M hydroxyurea</td>
<td>7.0 ± 2.0</td>
<td>30</td>
</tr>
<tr>
<td>$2 \times 10^{-3}$ M hydroxyurea</td>
<td>5.1 ± 1.8</td>
<td>49</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$ M hydroxyurea</td>
<td>4.8 ± 2.0</td>
<td>52</td>
</tr>
<tr>
<td>$10^{-3}$ M hydroxyurea</td>
<td>5.3 ± 1.3</td>
<td>47</td>
</tr>
<tr>
<td>No treatment</td>
<td>10.0 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

75,000 bone marrow cells cultured per plate. Means ± SD from seven plates/group.
All estimates are per femur (± SD). Six mice were used per group. Fifteen plates were counted per group to determine CFU-M and CFU-c levels; for regenerating marrow samples 7.5 x 10⁵ and 20 x 10⁵ cells, respectively, were cultured in 1.15 ml.

### Table 3. Effect of Hydroxyurea Treatment In Vivo on CFU-M and CFU-c Levels in Bone Marrow

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Total Cells*</th>
<th>CFU-M†</th>
<th>Total Cells</th>
<th>CFU-M</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(x 10⁻⁶)</td>
<td>(x 10⁻⁵)</td>
<td>(x 10⁻⁶)</td>
<td>(x 10⁻⁵)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13.3 ± 1.0</td>
<td>1575 ± 240</td>
<td>45.65 ± 2.89</td>
<td>10.5 ± 1.3</td>
<td>1355 ± 280</td>
</tr>
<tr>
<td>2</td>
<td>19.0 ± 1.6</td>
<td>2072 ± 202</td>
<td>44.29 ± 3.16</td>
<td>15.3 ± 2.1</td>
<td>1689 ± 244</td>
</tr>
<tr>
<td>3</td>
<td>22.5 ± 2.6</td>
<td>2925 ± 270</td>
<td>63.11 ± 5.94</td>
<td>20.0 ± 1.4</td>
<td>2460 ± 320</td>
</tr>
<tr>
<td>4</td>
<td>20.0 ± 1.1</td>
<td>2240 ± 320</td>
<td>52.14 ± 3.70</td>
<td>18.5 ± 1.8</td>
<td>2035 ± 240</td>
</tr>
<tr>
<td>5</td>
<td>23.3 ± 1.0</td>
<td>4534 ± 395</td>
<td>88.47 ± 8.56</td>
<td>23.4 ± 4.2</td>
<td>3475 ± 487</td>
</tr>
<tr>
<td>6</td>
<td>18.2 ± 2.3</td>
<td>2133 ± 230</td>
<td>43.42 ± 5.25</td>
<td>14.9 ± 1.0</td>
<td>1698 ± 103</td>
</tr>
</tbody>
</table>

*All estimates given per femur (± SD) based on assays performed on bone marrow cells taken from three animals/group.
†CFU-M levels determined from 15 or 16 plates/group.
§CFU-c estimates made from three to nine plates/group.
§ Reduction percentage calculated as (1-hydroxyurea/control) x 100.

Cells per femur were obtained from mice after hydroxyurea treatment than were obtained from untreated animals. This small reduction in the number of CFU-M observed after administration in vivo was not consistent with the observations in vitro. In a separate experiment, the effect of treating marrow cells either in suspension culture or in the intact animal was compared (Table 4). Hydroxyurea given in vivo did not influence the incidence of CFU-M, but a 50% reduction was observed by incubating isolated marrow cells for 60 min in the presence of 2 x 10⁻³ M hydroxyurea. In this experiment, the proportion of CFU-c killed was independent of whether the hydroxyurea treatment was in vivo or in vitro.

To determine if some cell cycle regulatory mechanism was operating in the animal, the effect of hydroxyurea on rapidly cycling marrow cells was studied.

### Table 4. Comparison of the Sensitivity of Marrow CFU-M to Hydroxyurea In Vivo and In Vitro

<table>
<thead>
<tr>
<th>Hydroxyurea In Vivo</th>
<th>Hydroxyurea In Vitro</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Treated</td>
<td>In Vivo</td>
</tr>
<tr>
<td>CFU-M/plate</td>
<td>8.2 ± 0.8</td>
<td>8.3 ± 1.2</td>
</tr>
<tr>
<td>CFU-c/plate</td>
<td>70.0 ± 5</td>
<td>48.0 ± 6.2</td>
</tr>
</tbody>
</table>

75,000 and 30,000 cells cultured/plate (1 ml) for CFU-M and CFU-c assays, respectively. Means ± SD from nine plates.

### Table 5. Comparison of the Reduction in CFU-M Levels in Normal and Reconstituted Mice After Hydroxyurea Treatment

<table>
<thead>
<tr>
<th>Exp. Group</th>
<th>Total Cells (x 10⁻⁶)</th>
<th>CFU-M (x 10⁻⁵)</th>
<th>Total Cells (x 10⁻⁶)</th>
<th>CFU-M (x 10⁻⁵)</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>13.3 ± 1.0</td>
<td>1575 ± 240</td>
<td>45.65 ± 2.89</td>
<td>10.5 ± 1.3</td>
<td>1355 ± 280</td>
</tr>
<tr>
<td>10-day</td>
<td>6.3 ± 0.8</td>
<td>353 ± 104</td>
<td>9.00 ± 1.33</td>
<td>6.6 ± 1.2</td>
<td>178 ± 77</td>
</tr>
</tbody>
</table>

All estimates are per femur (± SD). Six mice were used per group. Fifteen plates were counted per group to determine CFU-M and CFU-c levels, for regenerating marrow samples 7.5 x 10⁶ and 20 x 10⁶ cells, respectively, were cultured in 1.15 ml.
Mice were lethally irradiated (900 R) and reconstituted with the cells of half a femur from a syngeneic donor. After either 7 or 10 days, six reconstituted mice and six normal mice were injected with hydroxyurea, and 3 hr later the marrow cells were taken, pooled, and assayed for CFU-M and CFU-c content. Uninjected mice were used as controls.

The number of CFU-M and CFU-c in regenerating marrow was markedly lower in the femurs of normal adult mice (Table 5). For these experiments 75,000 and 200,000 cells were plated for CFU-c and CFU-M assays, respectively. The population sizes of CFU-M and CFU-c in regenerating marrow after hydroxyurea administration were 50% lower than in untreated control animals (Table 5). In contrast to this finding, little difference was observed between the femoral content of CFU-M in normal mice treated with hydroxyurea and the uninjected controls.

**DISCUSSION**

The clonable megakaryocyte progenitor cells were previously shown to give rise to clones of up to 20-30 cells (modal values 6–12 cells), indicating that the proliferating compartment is capable of at least four or five cell divisions prior to endomitosis. Clones of up to five to ten times that size (250-300 acetylcholinesterase-positive cells) are reported in this communication, indicating that the megakaryocyte progenitor cells are capable of extensive cell division prior to endomitosis and cytoplasmic maturation to platelet formation.

It is noteworthy that the number of cells per clone was bimodally distributed with peak values differing by a factor of 2. The distribution may reflect differences in endomitosis versus proliferation within the differentiation cells that make up the clone, since the clones with higher numbers of acetylcholinesterase-positive cells generally contained small cells that could not be readily identified directly in the plate as belonging to the megakaryocyte cell lineage (Fig. 1). Penington and co-workers suggested that the endomitosis may influence the differentiation pattern in that megakaryocytes with a lower ploidy (8n) give rise to larger and denser platelets, which are functionally different from the small, less dense platelets released from megakaryocytes with higher ploidy values (32n).

Megakaryopoiesis may also be regulated in part by the cell cycle status of the CFU-M compartment. It is generally agreed that with the present cloning efficiency there are at least 2000-5000 CFU-M/femur (10-25 CFU-M/10⁵ bone marrow cells). Megakaryocytes are found in mouse bone marrow with a frequency of approximately 0.07%, or 15,000 megakaryocytes/femur. The numbers of morphologically identifiable megakaryocytes are therefore at most only three- to eightfold greater than the number of megakaryocyte progenitor cells. Based on colony size, the number of recognizable megakaryocytes should be at least 10–200 times greater than the number of CFU-M present per femur. It is possible that the CFU-M have a certain capacity for self-renewal, while the remainder differentiate into megakaryocytes. Long-term bone marrow liquid culture experiments indicate, however, that the CFU-M compartment does not have a great capacity for self-renewal.

The maintenance of CFU-M as a slowly cycling population could be controlled by a local feedback mechanism involving marrow stromal elements or...
the marrow population of maturing megakaryocytes. This mechanism may not be operative, however, when the progenitor cells are removed from the marrow environment. Whatever the mechanism, it must be reversed within 30 min, the length of time of the incubation for $^3$H-Tdr incorporation experiments. This time interval is similar to that reported for the reversibility of inhibitory action on other hemopoietic cell lineages.$^{14}$

While these inhibitors do not appear to affect the expansion and differentiation of their respective progenitor cells,$^{25-27}$ these activities do modulate late events of cell division and maturation, stages of differentiation that may be condensed into endomitosis and cytoplasmic maturation within the megakaryocyte cell pool. By analogy, platelet and platelet extracts may influence these terminal stages of maturation and platelet release, whereas control of proliferation and differentiation of megakaryocyte precursor cells could be subject to different regulatory mechanisms.

Study of the incidences, cell cycle, and proliferative capacity of megakaryocyte progenitor cells that clone in vitro indicates that megakaryopoiesis may not be regulated simply by a single stimulator (thrombopoietin) and a single platelet-derived inhibitor. Control may be achieved by a sensitive balance between several stimulators governing proliferation, endomitosis, and cytoplasmic maturation and feedback mechanisms, systemically via platelet levels and possibly locally by a narrow feedback mechanism operative via a cell blockade of the committed progenitor cell compartment.

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REFERENCES

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