Immature Megakaryocytes in the Mouse: Physical Characteristics, Cell Cycle Status, and In Vitro Responsiveness to Thrombopoietic Stimulatory Factor

By Michael W. Long, Neil Williams, and Shirley Ebbe

The heterogeneity among immature megakaryocytes has been examined by physical properties, cell cycle status, and responsiveness to thrombopoietic stimulatory factor. Three types of immature megakaryocytes exist that can be recognized by acetylcholinesterase staining, nuclear shape, high nucleus/cytoplasm ratio, and small size (8–18 μm) with respect to mature megakaryocytes (>18 μm). These three acetylcholinesterase-containing cell types are distinguished by their nuclear configuration: a round, indented, and lobed nucleus. The lobed cell type was found to overlap with and enhance detection of megakaryoblasts (stage I megakaryocytes). These cells had a sedimentation velocity range of 3.5–19.0 mm hr⁻¹ and a density range of 1.072–1.095 g cm⁻³. Separation of these three classes of immature megakaryocytes was achieved by equilibrium density centrifugation with modal buoyant densities of 1.079 g cm⁻³ (round), 1.084 g cm⁻³ (indented), and 1.089 g cm⁻³ (lobed). In the presence of thrombopoietic stimulatory factor, the round nucleated cells, but not the indented or lobed nuclei morphology, were observed to develop into large mature megakaryocytes in 60-hr semisolid cell cultures. Development of two cell groups, or colonies of megakaryocytes, was not observed during this in vitro incubation period. In vivo treatment with hydroxyurea indicated that 57.5% ± 19% of the round nucleus form were actively synthesizing DNA. No reduction in the numbers of indented or lobed nucleus forms were observed following hydroxyurea treatment. The data in this report strongly support the concept that these three types of immature megakaryocytes reflect the early maturation stages occurring in megakaryocyte differentiation.

IN MURINE AND HUMAN megakaryocytosis there is a group of cells that may mark the transition in differentiation between the committed progenitor cell and mature megakaryocyte compartments. These cells are among the earliest of detectable megakaryocytes. They have been shown to bear megakaryocyte-platelet distinct markers, are markedly smaller than mature megakaryocytes (8–18 μm), have a higher nucleus/cytoplasm ratio, and in certain species contain low amounts of acetylcholinesterase. Immature murine megakaryocytes have been observed using a combination of acetylcholinesterase staining and phase contrast microscopy, and classes of these smaller cells included round, indented, and lobulated nuclear configurations. Moreover, progressive increases in size and acetylcholinesterase content were noted within these classes of immature megakaryocytes. These findings strongly suggested that the three morphological classes of early differentiating megakaryocytes may represent separate maturation stages within the immature acetylcholinesterase-positive megakaryocytes. A subpopulation of these cells (identified by light microscopy) has been separated from the bone marrow by velocity sedimentation. Unlike the megakaryocyte progenitor cell (CFU-MK), immature megakaryocytes did not form megakaryocyte colonies. Rather, in the presence of thrombopoietic stimulatory factor, they developed into single mature megakaryocytes.

The heterogeneity among these immature megakaryocytes was examined to further describe their properties and their relationships to the progenitor cells (CFU-MK) and mature megakaryocytes. Changes in enzyme content and cell size among these cells have been reported. Bone marrow cells were separated by velocity sedimentation and buoyant density centrifugation. Various subsets of these early differentiating megakaryocytes were characterized by their cell cycle status and relative responsiveness to thrombopoietic stimulatory factor.

MATERIALS AND METHODS

Mice
C57BL/6 (Cumberland View Farms) or C3H mice (Charles River) were used.

Quantification of Immature Megakaryocytes
Bone marrow cells were suspended in 10.0 ml of CATCH media. One-hundred microliters of the bone marrow suspension (containing 1–3 × 10⁵ nucleated cells) were loaded into the cytocentrifuge sample chamber and spun for 3 min at 40 g. The chamber was then washed with 200 μl fresh CATCH and respun for 10 min at 40 g.

From the Department of Hematopoietic Development, Sloan-Kettering Institute for Cancer Research, Rye, N.Y. and the Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, Calif. Supported in part by Grants HL 22451, CA 08748, and AM 21355 from NIH; CH3E from the American Cancer Society and Gar Reichman Foundation; and the Office of Health and Environmental Research of the U.S. Department of Energy under contract W-7405-ENG-48, M.L. is a Leukemia Society of America Special Fellow. N.W. is a Leukemia Society of America Scholar. Submitted June 4, 1981; accepted November 5, 1981.

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0006-4971/82/5903-0017$01.00/0

Blood, Vol. 59, No. 3 (March), 1982
The cells thus prepared were heat fixed and incubated in acetylthiocholine substrate for 6–6.5 hr. They were fixed and counterstained in Harris hematoxylin and quantitated using phase contrast microscopy. Three morphological types of small acetylcholinesterase-positive early megakaryocytes were detected. One of the salient distinguishing features of these cells was nuclear shape; hence, they are referred to as the round, indented, or lobulated form. The frequency of mature megakaryocyte stages was determined by conventional staining techniques as reported elsewhere.

**Cell Separation**

Velocity sedimentation was performed at unit gravity according to the method of Miller and Phillips. Bone marrow was obtained from 10–15 mice by flushing with cold (4°C) PBS. The cells were kept on ice during collection and the separation was carried out at 4°C. Following sedimentation for 2–3 hr, 35-ml fractions were collected and the cells harvested by centrifugation at 400 g for 7 min. The cells were resuspended in serum-free CATCH media. Cells were then cytacentrifuged and stained for acetylcholinesterase or May-Grünwald-Giemsa quantification. In certain experiments these cells were resuspended in serum-free McCoy’s 5A (SK1) and tested for responsiveness to thrombopoietic stimulatory factor.

For density gradient separation, bone marrow cells were separated on continuous gradients of 25%–35% bovine serum albumin (BSA, fraction V, Sigma Chemical Co., St. Louis, Mo.), prepared at mouse tonicity (310 mOsM), pH 5.1415 Gradients were spun at 4000 g for 30 min. Fractions were collected and the density of each fraction determined. All steps were performed at 4–6°C. Cell counts of each fraction were determined using a hemocytometer. Cells harvested were prepared for cytocentrifugation or in vitro growth. All data were calculated as cells/fraction/density increment to correct for nonlinearity of the gradient. All plots presented were normalized as a percent of peak fraction.

**In Vitro Growth of Immature Megakaryocytes**

Pooled fractions from the above separations (containing $10^7–10^8$ immature megakaryocytes) and 250 μg of thrombopoietic stimulatory factor (prepared from a human embryonic kidney cell line; a generous gift by Dr. I. P. McDonald, University of Tennessee Memorial Research Center) were added to 1.0 ml of McCoy’s 5A (SK1) containing 0.25% bacto-agar (Difco, Detroit, Mich.). The media used were modified as reported by Williams et al. for the growth of megakaryocyte progenitor cells (CFU-Mk), except that PGE$_2$ was omitted. Cells were incubated at 37°C for 60 hr, removed, dried in situ, and stained for acetylcholinesterase. Single megakaryocytes were counted at 90x magnification.

**Sensitivity of Immature Megakaryocytes to Hydroxyurea**

The proportion of early differentiated megakaryocytes and megakaryocyte progenitor cells (CFU-Mk) in S phase of DNA synthesis was determined by intraperitoneal injection of hydroxyurea (900 mg/kg) into C57BL/6 mice (males, Cumberland Farms). The mice were sacrificed 3 hr after injection, and the femurs were excised and flushed into 10.0 ml of CATCH media. Cell suspensions were cytacentrifuged as described and morphological classes of immature megakaryocytes quantified as previously reported. Control animals received equal volumes of PBS. A total of 50 individual animals (25 controls and 25 hydroxyurea-treated animals) were examined in two sets of experiments. Megakaryocyte progenitor cell (CFU-Mk) assays were performed as described elsewhere.

**RESULTS**

**Velocity Sedimentation Profile of Immature Murine Megakaryocytes**

The sedimentation profiles of immature murine megakaryocytes exhibiting acetylcholinesterase megakaryocytes and total nucleated marrow cells are depicted in Fig. 1. These small immature megakaryocytes had a sedimentation rate ranging from 3.5 to 19.0 mm hr$^{-1}$. The profile showed three regions with two peaks at 5.7 mm hr$^{-1}$ and 7.9 mm hr$^{-1}$, and a pronounced shoulder was noted at 10.0 mm hr$^{-1}$. The total recovery of all cell types (i.e., total nucleated and immature megakaryocytes) ranged between 70% and 80%. Mature megakaryocytes were not plotted due to their low recovery (<50%) since they sediment at 33–100 mm hr$^{-1}$.

The distributions of the three morphological types of
immature megakaryocytes are shown in Fig. 2. All three cell types had a peak sedimentation rate of 5.7 mm hr$^{-1}$. Cell profiles of the indented and lobed nuclear configuration had the broadest distributions, with shoulders at 8.3 and 9.0 mm hr$^{-1}$, respectively. Differential analysis of the areas under the curves from linear plots (data not shown) indicated that the lobulated nucleus morphology accounted for the majority of acetylcholinesterase-positive cells that sedimented at 7.9 mm hr$^{-1}$ in Fig. 1.

**The Immature Megakaryocyte Compartment as Detected by Acetylcholinesterase or Romanovsky Staining**

Apparent discrepancies existed between known frequencies of mature megakaryocytes identified by Romanovsky staining and the frequencies observed when using the combination of acetylcholinesterase and phase contrast. Examination of the relative percentages of the mature and immature murine bone marrow megakaryocytes determined by Romanovsky staining is shown in Table 1. These data show that a high proportion of megakaryocytes are mature cells (stages II and III). However, it has been reported that use of acetylcholinesterase in conjunction with phase contrast results in lower numbers of mature megakaryocytes and increased numbers of immature megakaryocytes. Also, similarities in size and nuclear shape were seen between these two populations. It was possible that the two staining techniques were analyzing the same cell population (i.e., megakaryoblasts) but differing in sensitivity.

A velocity sedimentation experiment was performed in which duplicate slides from each fraction were prepared, and the cells stained for either acetylcholinesterase activity or with May-Grünewald-Giemsa. The slides were then analyzed for the presence of the lobulated type of immature megakaryocytes or for megakaryocyte morphology (stages I-III; Ebbe$^9$), respectively.

The results of these experiments are shown in Fig. 3. Megakaryoblasts (stage I megakaryocytes) sedimented with a rate very similar to acetylcholinesterase-positive cells with the lobulated nuclear morphology. This suggested that megakaryoblasts are the predominant cell type within the megakaryocyte lineage occurring at this sedimentation velocity. Calculation of the relative areas under the curves (using linear graphs) indicated that megakaryoblasts (and promegakaryocytes) account for 64% of all lobulated-type acetylcholinesterase-positive cells (data not shown). The remaining numbers of the lobed form of immature megakaryocytes are, presumably, megakaryoblasts. Thus, the acetylcholinesterase technique primarily improves quantitation of megakaryoblasts by allowing the detection of cells that are generally difficult to classify as megakaryocytes by Romanovsky staining.

**Buoyant Density Characteristics of Immature Acetylcholinesterase-Containing Megakaryocytes**

The density distribution for the total population of immature megakaryocytes ranged from 1.072 g cm$^{-3}$ to >1.095 g cm$^{-3}$ (Fig. 4, upper panel). The three morphological types showed separate but overlapping

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**Table 1. Proportions of Mature Megakaryocytes in the Mouse**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Percent ± SEM</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I (megakaryoblast)</td>
<td>17.7 ± 1.0</td>
<td>12.4–28.4</td>
</tr>
<tr>
<td>Stage II (promegakaryocyte)</td>
<td>32.0 ± 0.9</td>
<td>23.6–39.2</td>
</tr>
<tr>
<td>Stage III (megakaryoblast)</td>
<td>50.3 ± 1.5</td>
<td>36.0–66.2</td>
</tr>
</tbody>
</table>

Megakaryocyte frequencies determined by Romanovsky staining. Percent values are mean ± SEM determined from 250 megakaryocytes per mouse ($n = 24$).
density distributions. The modal density values for the round nucleus (1.079 g cm⁻³), indented nucleus (1.084 g cm⁻³), and lobulated nucleus (1.089 g cm⁻³) morphologies were obtained. The large mature megakaryocytes had a modal density >1.095 g cm⁻³. Cell recovery in these gradients was 38% for total nucleated cells and 82% for immature megakaryocytes.

**Responsiveness of Immature Megakaryocytes to Thrombopoietic Stimulatory Factor**

The cells that responded in vitro to thrombopoietic stimulatory factor (bottom panel of Fig. 4) were found in a density range that closely corresponds to that of the round form of immature megakaryocyte (1.072–1.084 g cm⁻³). No response to thrombopoietic stimulatory factor was seen in the region where cells of the indented and lobulated shaped nucleus were observed. As reported previously, growth of immature acetylcholinesterase-containing megakaryocytes does not occur in the absence of thrombopoietic stimulatory factor.⁴

The response of immature megakaryocytes harvested from velocity sedimentation experiments is seen in Fig. 5. Pooled fractions of these megakaryocytes sedimenting at 5.0–6.5 mm hr⁻¹, 8.5–9.5 mm hr⁻¹, and
The responsiveness of immature megakaryocytes separated by velocity sedimentation to thrombopoietin stimulatory factor. Cells sedimenting over the ranges given were pooled and tested for responses in vitro (see Materials and Methods). Values are means ± SD of 4 or 5 separate experiments in which 3-5 replicate cultures were performed for each of the pooled fractions.

11.0-16.0 mm hr\(^{-1}\) were tested. No difference in plating efficiency was seen between cells settling at 8.5-9.9 mm hr\(^{-1}\) and those with a sedimentation velocity of 11.0-16.0 mm hr\(^{-1}\). The fraction of cells with a peak sedimentation velocity range of 5.0-6.5 mm hr\(^{-1}\) showed a decreased responsiveness to thrombopoietic stimulatory factor on a per cell basis.

**Sensitivity of Immature Megakaryocytes to Hydroxyurea**

The results of in vivo hydroxyurea treatment on immature megakaryocytes are given in Table 2. Administration of hydroxyurea resulted in a 57.5% ± 19% (mean ± SD) reduction in those immature megakaryocytes with the round nucleus morphology and no reduction in the intended or lobulated cell types. By contrast, megakaryocyte progenitor cells (CFU-Mk) were reduced by in vivo administration of hydroxyurea, indicating a 10% ± 13% reduction in cell numbers per femur, a result in agreement with previous results.\(^{20,21,27}\)

**DISCUSSION**

The immature megakaryocytes in the mouse have been shown to be comprised of three distinct morphological subsets, based on acetylcholinesterase staining and phase contrast microscopy. These cells differ in size, nuclear conformation, and acetylcholinesterase content.\(^a\)

The populations of immature megakaryocytes and megakaryoblasts were found to be markedly similar in their sedimentation characteristics. It was calculated that 64% of the cells described as having a lobulated nucleus (using acetylcholinesterase staining) corresponded with megakaryoblasts or promegakaryocytes using Romanovsky staining criteria. Therefore, the megakaryoblast and lobulated form of immature megakaryocyte are the same cell population detected by two separate techniques. Clearly, the change from undetectable megakaryoblast to detectable megakaryoblast occurs within the lobed cell compartment. The proportion of megakaryocytes calculated using a combination of these two techniques is seen in Table 3.

Immature megakaryocyte types were not segregated by their sedimentation rate. These cells overlap with but are separable from the peak of megakaryocyte progenitor cells\(^{15,20,22}\) and from the rapidly sedimenting more mature megakaryocytes.\(^8\) The overall sedimentation range (3.5-19.0 mm hr\(^{-1}\)) differs from our previous report (8-19 mm hr\(^{-1}\)).\(^4\) The discrepancy in these findings is from the increased sensitivity of detection of acetylcholinesterase-positive cells by using phase contrast microscopy. The three morphological types did have distinctly differing modal buoyant densities (Table 2). These cells show a density range that is intermediate between the megakaryocyte progenitor cells\(^{15,23}\) and mature megakaryocytes.\(^23,24\) The density range of the immature megakaryocytes differed from the range previously reported.\(^23\) In contrast to that study, the majority of the immature mega-

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**Table 2. Characteristics of Murine Femoral Megakaryocyte Compartment**

<table>
<thead>
<tr>
<th>Sedimentation velocity (mm hr(^{-1}))</th>
<th>CFU-Mk*</th>
<th>Immature Megakaryocytes</th>
<th>Mature Megakaryocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5-7.5</td>
<td>3.5-7.5</td>
<td>3.5-8.3</td>
<td>3.5-19.0</td>
</tr>
<tr>
<td>Modal buoyant density (g cm(^{-3}))</td>
<td>&lt;1.075</td>
<td>1.079</td>
<td>1.084</td>
</tr>
<tr>
<td>S phase (% reduction)</td>
<td>10 ± 13</td>
<td>57.5 ± 19</td>
<td>0</td>
</tr>
<tr>
<td>TSF response</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*Megakaryocyte progenitor cell (CFU-Mk) data except percent in S phase taken from Williams et al.\(^{22,26}\) and Freedman et al.\(^{27}\) N/A, not applicable; NT, not tested.
The percentages of the megakaryocyte compartment were recalculated on the basis that 64% of the lobed cells were judged to belong to the population of recognizable megakaryocytes. The calculations were rounded off to the nearest 0.5%, resulting in the 1% discrepancy in the table. Romanovsky data derived from Table 1 and Fig. 4. Acetylcholinesterase data from Long and Williams; percentages are: round nucleus cell type = 1.5%, indented nucleus = 3.4%, lobed nucleus = 68.5% ± 2%, mature = 28.5% ± 2%.

In vitro cultivation of fractions enriched for each cell type (separated by density centrifugation) indicated that the round nucleus morphology, but not the indented or lobulated cell types, responded to thrombopoietic stimulatory factor (Fig. 4). Assuming that increasing nuclear complexity and enzyme content reflect maturation changes, then this finding shows that the earliest detectable cell among megakaryocytes is capable of responding to regulatory signals. The lack of an observed in vitro response of the indented or lobulated forms is due either to an inability of the cells to respond in vitro or results from an altered responsiveness of these cells to stimuli. In vivo evidence has shown that the recognizable megakaryocyte compartment (Romanovsky staining) responds to thrombopoietic stimulatory factor preparations and alterations in platelet mass. The reason for the lack of in vitro response in the population of lobulated immature megakaryocytes is unclear.

In vitro administration of hydroxyurea resulted in a 57.5% reduction of the round nucleus form of immature megakaryocyte and no change in numbers of indented or lobulated morphologies (Table 2), showing that in addition to size, nucleus/cytoplasm ratio, enzyme content, and density characteristics, the proportion of cells in DNA synthesis allows further distinction among these three cell types.

The hydroxyurea and thrombopoietic stimulatory factor experiments indicate that a great deal of DNA synthesis is occurring in a cell type (round nucleus) that has apparently lost its proliferative potential. Therefore, it may be the earliest detectable cell in the megakaryocytic sequence that has the ability to endoreduplicate. Ebbe and Feinendegen previously suggested that unrecognizable megakaryocyte precursors actively synthesize DNA.

In summary, three morphological types of immature megakaryocytes differ in their physical properties, in vitro responsiveness to thrombopoietic stimulatory factor, and amount of DNA synthesis. All these parameters strongly support the concept that these types of immature megakaryocytes reflect sequential changes in early megakaryocyte differentiation.

ACKNOWLEDGMENT

This work was done within the Department of Developmental Hematopoiesis headed by Dr. M. A. S. Moore. The authors are indebted to him for careful review of the study. The authors would also like to thank Dr. Ted McDonald for his generous gift of thrombopoietic stimulatory factor.

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IMMATURE MEGAKARYOCYTES

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