Characterization of Megakaryocyte Spleen Colony-Forming Units by Response to 5-Fluorouracil and by Unit Gravity Sedimentation

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Properties of megakaryocyte progenitor cells in mouse bone marrow have been examined using an in vivo assay system. Perturbation with 5-fluorouracil (5-FU) and separation by unit gravity sedimentation was used to characterize the cells. Bone marrow was assayed for the presence of megakaryocyte colony-forming cells (MK CFU-S) by transplantation into lethally irradiated mice and examining spleen sections 10 days later. Donor mice were untreated or injected intravenously with 5-FU (150 mg/kg), 1 (FU-1) or 7 (FU-7) days beforehand. There was a lack of correlation between the numbers of MK CFU-S and cells giving rise to macroscopic spleen surface colonies (CFU-S10). The sedimentation profile of MK CFU-S in normal marrow was similar (modal velocity 4.16 ± 0.05 mm/hr) to that of CFU-Sp. In FU-1 marrow, MK CFU-S exhibited a bimodal sedimentation profile, with peaks at 3.26 ± 0.06 mm/hr and 4.53 ± 0.07 mm/hr. The marrow content of CFU-S10 was reduced to 5% of normal, while MK CFU-S numbers were only reduced to 60%. In FU-7 marrow, the sedimentation profile of MK CFU-S (modal velocity 4.86 ± 0.16 mm/hr) differed from that of CFU-Sp (5.5 ± 0.16 mm/hr). It was concluded MK CFU-S and CFU-S10 are different entities. The MK colonies formed from FU-1 marrow contained on average 3.8-fold more cells than those formed from normal marrow. The enhanced megakaryocyte production may be accounted for on the basis of the generation-age model for cell proliferation. It is proposed that MK CFU-S are a heterogeneous population with regard to proliferation potential and that the FU-1 marrow contains cells that survive 5-FU and have a high proliferative potential. These cells may be equivalent among megakaryocytic progenitors to the high proliferative potential colony-forming cells of the granulocyte/macrophage series. They may be responsible for the enhanced megakaryocytopenias seen in the marrow of mice 7 days after the injection of 5-FU.

MEGAKARYOCYTE PROGENITOR cells are morphologically unidentifiable. Their existence has been inferred from experiments such as "flash" labeling of megakaryocytes with tritiated thymidine,1 differential effects of radiation1 and of cyclespecific cytotoxic agents,3,4 and culture of megakaryocytes from murine marrow cells in conditioned media.5-7 The known properties of these cells have been reviewed by Williams.8 Recently, it has been observed that a marked rebound megakaryocytopenia occurs in mouse bone marrow 7 days after the administration of the antineoplastic drug, 5-fluorouracil (5-FU).9,10 It has also been demonstrated in transplantation studies that for doses producing equivalent numbers of spleen surface colonies, as assayed by the method of Till and McCulloch,11 there are many more megakaryocytes in spleen sections of recipients of marrow from 5-FU-pretreated mice than recipients of normal marrow.12,13 These observations suggested that pretreatment of mice with 5-FU could help to elucidate the properties of megakaryocyte progenitors that are assayable in vivo. The cells ("units") responsible for generating megakaryocyte colonies in the spleen of irradiated recipients will be referred to as megakaryocyte colony-forming units, spleen (MK CFU-S). In the present study we have compared MK CFU-S of normal marrow with those obtained from 1 day post 5-FU (referred to as FU-1) and 7 day post 5-FU (referred to as FU-7) treated mice. To further characterize MK CFU-S, the marrow were separated into fractions by velocity sedimentation, and the MK CFU-S content of the fractions assayed by transplantation into lethally irradiated syngeneic mice.

MATERIALS AND METHODS

In each experiment, 3-mo-old BALB/c mice were divided into groups of 5. Some groups received an intravenous injection of 5-FU (150 mg/kg body weight) and were sampled 1 or 7 days later; others were left untreated. The mice were killed by cervical dislocation under ether anesthesia, and bone marrow cells were harvested aseptically from femurs by aspiration with a 21-gauge needle into 5 ml of balanced salt solution (BSS), supplemented with 3% of new-born calf serum (NCS). Single-cell suspensions were prepared by gentle pipetting.

Unit gravity sedimentation was carried out as described by Miller and Phillips,14 using a 11.5-cm diameter chamber (Staput, Johns Scientific, Toronto, Canada). Prior to sedimentation, cell counts were made using a hemocytometer to determine the total number of cells to be loaded, as the ratio of nucleated cells to erythrocytes differed significantly in the marrow of the 3 groups of mice. Only 6.3% ± 0.3% of FU-7 marrow cells were nucleated, compared with 38.0% ± 4.6% and 60.0% ± 5.3% for FU-1 and normal marrow, respectively. To avoid streaming, the total number of cells loaded did not exceed 2 × 10⁶ cells in any experiment. Cells were layered on a gradient of 15%–30% NCS in BSS and allowed to sediment for 3.5 hr at 4°C. Twenty-five-milliliter fractions were collected and centrifuged at 200 g for 10 min at 4°C. The cell pellets were each
resuspended in 2 ml of BSS plus 3% NCS and the nucleated cell content determined. Cell suspensions of fractionated and unfractio-
ated bone marrow were then diluted with BSS plus 3% NCS and
injected intravenously into recipient mice in 0.5-ml aliquots contain-
ing 2 x 10⁶ cells (normal, FU-1) or 2 x 10⁶ cells (FU-7). Groups of
10 lethally irradiated (7.5 Gy Philips 250 kVp 15 ma, filtration 0.25
mm Cu, 1 mm Al), syngeneic mice were used for each assay.

Ten days after transplantation, the mice were killed and spleens
removed and fixed in formal acetic ethanol for 2 hr. followed by 10%
neutral formalin. Surface colonies were counted using a dissecting
microscope at 16 x magnification. The spleens were then processed
for histologic examination. Midline sections, 3 µm thick, were cut
from wax-embedded material and stained with hematoxylin and
eosin. Clearly recognizable megakaryocytes (containing a nucleus)
were counted at 500 x magnification. Megakaryocyte colonies were
also scored on the sections. Any single isolated megakaryocyte, or a

group of these cells was scored as a spleen megakaryocyte colony if
separated by a distance of at least 100 µm from other megakary-
cytes. No distinction was made if megakaryocytes were found among
cells of a colony of another type. The diameter of megakaryocytes
was measured with an eye-piece micrometer. Areas of spleen
sections were determined by tracing the outline of a 10 x magnified
image of the section projected onto a Hewlett Packard Digitizing
Tablet, and feeding the data to an HP 85 microprocessor. Previous
studies have shown that there is a linear relationship between
megakaryocytes per section and marrow dose injected up to 140
MK/sec.²

RESULTS

Cell Sedimentation Profiles

Unit gravity sedimentation of suspensions of normal and FU-1 bone
marrows yielded a bimodal distribution of nucleated cells. In contrast, the profile obtained for
FU-7 marrow cells was unimodal, as previously
described.¹⁰ The mean peak velocities of the normal
nucleated cell profile were 2.9 mm/hr and 5.0 mm/hr, while the profile of FU-1 marrow had peaks at 3.1
mm/hr and 4.5 mm/hr. The FU-7 marrow suspension
yielded a single peak at 3.4 mm/hr. Total nucleated cell recoveries for normal, FU-1, and FU-7 marrows
following unit gravity sedimentation were 50.8% ±
8.5%, 68.3% ± 5.7% and 48.4% ± 9.1%, respectively.

Mean Diameter of MK

The diameters of nucleated megakaryocytes chosen
at random were measured on spleen sections with a
calibrated eye-piece graticule. More than 150 cells
were measured for each of the 3 treatment schedules,
following transplantation of unfractioinated marrow.
There was a small, but significant, increase (p < 0.01)
in the mean diameter of megakaryocytes in spleens of

*The mean area of spleen sections was 20 sq mm, which is
equivalent to a total of 637 “counting fields” of radius 100 µm. The
probability of two unrelated MK occurring in the same “counting
field” by chance can be estimated. If the average number of
megakaryocytes per section is 10, the probability would be 0.00025.
For an average of 50 MK per section, the probability would be
0.006.

mice injected with FU-7 marrow compared with those
injected with normal or FU-1 marrow (Table 1). However, the increase was not such as to markedly
affect the magnitude of the correction factor,¹⁵ which
must be taken into account when comparing the
observed frequencies of megakaryocytes in sections to
allow for differences in cell diameters between popula-
tions. The change in size, which is equivalent to a 50%
increase in volume compared to normal, may reflect
increased endomitosis among a proportion of the cells,
but DNA measurements to confirm this have not been
carried out.

Sedimentation Profiles of MK CFU-S

Assay of the different fractions of bone marrow
from normal and FU-7 mice consistently (4 experi-
ments each) produced a unimodal distribution of MK
CFU-S (Fig. 1, A and C). For the fractions derived
from FU-1 marrow, however, there was a bimodal
distribution, with greater variation in the values at
different sedimentation velocities between experiments
(Fig. 1B). When the results of unit gravity sedimenta-
tion were plotted as MK/section or MK/sq cm section,
instead of MK colonies/section, the velocity sedimen-
tation profiles remained essentially unchanged. The
modal sedimentation velocity of the fraction that pro-
duced the most MK colonies was 4.16 ± 0.05 mm/hr
for normal marrow. For FU-7 marrow the value was
higher, at 4.86 ± 0.16 mm/hr, possibly reflecting cells
that are in cycle, since the marrow is actively regener-
at ing at this time. For the FU-1 marrow, peaks were
observed at 3.26 ± 0.06 mm/hr and 4.53 ± 0.07 mm/hr.
In all cases, all the peak values were below the
mean peak velocity at which recognizable immature
MK sediment.¹⁸

The number of MK CFU-S/10⁵ nucleated cells
(Table 2) in FU-7 marrow was 34-fold greater than in
normal bone marrow (range 17–51-fold). In FU-1
marrow, the concentration was lower (0.6–1.0-fold).
Unit gravity sedimentation resulted in significant
enrichment of MK CFU-S only in normal marrow,
where an increase of 3-fold was obtained (Table 2).
MK colonies per section was the average of 7.6 MK, while FU-7 and normal marrow contained significantly more MK than those produced from spleen MK colonies (Fig. 2) derived from this material. The total number of MK CFU-S per femur was, however, not increased above normal (Table 2). The number of MK CFU-S was least in the FU-1 marrow, the most MK colonies, producing the number of CFU-S10 in normal marrow have 4 or fewer megakaryocytes, while for FU-1 marrow, over 50% of colonies contain more than 4 MK.

**Megakaryocytes per Colony in Spleen Sections**

Although the concentration (per 10^5 marrow cells) of MK CFU-S was least in the FU-1 marrow, the spleen MK colonies (Fig. 2) derived from this material contained significantly more MK than those produced by either FU-7 donor marrow or normal marrow (Table 2). FU-1 marrow produced colonies containing an average of 7.6 MK, while FU-7 and normal marrow produced colonies with an average of 3.0 and 2.0 MK, respectively. It will be appreciated that these differences are accentuated when the 3-dimensional nature of a colony is taken into consideration. The largest colony seen on a section contained 67 MK, which would suggest that the entire colony was probably composed of several hundred megakaryocytes.

A cumulative plot of percentage of colonies up to a certain size (plotted as logit versus 1n MK/colony) is shown in Fig. 3. The values gave three parallel lines for normal, FU-1 and FU-7 marrow. The arithmetic means of MK/colony for all 3 groups was situated at about the 73% point. It can be seen that 88% of colonies derived from normal marrow have 4 or fewer megakaryocytes, while for FU-1 marrow, over 50% of colonies contain more than 4 MK.

**CFU-S10 Sedimentation**

The sedimentation profile of cells ("units") in normal bone marrow responsible for forming surface colonies on the spleen assayed 10 days after transplantation (CFU-S10) is in agreement with published values, with a modal sedimentation velocity of 4.16 ± 0.05 mm/hr (Fig. 1A). Although the velocity sedimentation patterns of MK CFU-S and CFU-S10 in normal marrow were similar (Fig. 1A), there was no correlation of MK colonies per spleen section with spleen surface colonies (Fig. 4). FU-1 marrow in the doses assayed (2 × 10^3 cells) produced on average less than one surface colony per spleen, and no sedimentation profile could be obtained. FU-7 marrow samples (Table 2), as previously reported, had a high concentration of CFU-S10, ranging from 62 ± 22 to 124 ± 10 per 2 × 10^5 cells. The total number of CFU-S10 per femur was, however, not increased above normal (Table 2). The velocity sedimentation profile of CFU-S10 in FU-7 marrow was unimodal, with a peak at 5.5 mm/hr, which was greater than that of the MK CFU-S (4.86 mm/hr) (Fig. 1C). The number of MK CFU-S was actually 4.1 (± 0.65 SE) times greater than the number of CFU-S10 in the fraction of FU-7 marrow producing the most MK colonies, while in the fraction

### Table 2. MK Colonies, MK/Colony, and Spleen Surface Colonies 10 Days After Transplantation of Normal, FU-1, or FU-7 Bone Marrow (Average of Four Experiments ± Standard Deviation)

<table>
<thead>
<tr>
<th>Donor Status</th>
<th>Nucleated Cells × 10^5/Femur</th>
<th>Marrow Sample Transplanted</th>
<th>MK Colonies/Section × 2 × 10^5 Cells</th>
<th>MK/Colony</th>
<th>Spleen Colonies/2 × 10^5 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>30.8</td>
<td>Unfractionated</td>
<td>4.7 ± 1.3</td>
<td>2 ± 0.09</td>
<td>14.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Modal</td>
<td>13.6 ± 1.0</td>
<td>2.9 ± 0.12</td>
<td>30.6 ± 2.3</td>
</tr>
<tr>
<td>FU-1</td>
<td>24</td>
<td>Unfractionated</td>
<td>3.7 ± 0.5</td>
<td>7.6 ± 1.5</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Modal</td>
<td>6.4 ± 1.5</td>
<td>8.9 ± 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>FU-7*</td>
<td>3.5</td>
<td>Unfractionated</td>
<td>159 ± 44</td>
<td>3.0 ± 0.13</td>
<td>101 ± 45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Modal</td>
<td>233 ± 150</td>
<td>3.0 ± 0.3</td>
<td>149 ± 19</td>
</tr>
</tbody>
</table>

*Modal refers to cells from the modal sedimentation fraction. For 65 mice that did not receive a marrow transplant after irradiation, the mean number of MK colonies per section was 0.4, and spleen surface colonies was 0.2 after 10 days.

*The actual dose of FU-7 marrow injected was 2 × 10^5 cells.
Cells (designated MK CFU-S) are present in the bone marrow, which, when injected into lethally irradiated hosts, give rise to megakaryocyte colonies in the spleen. The nature of these cells has been explored by observing their response after perturbing the marrow with 5-FU and by using unit gravity sedimentation to characterize them. These cells exhibit different properties to those giving rise to macroscopic colonies in the spleen (CFU-S10). In spleens of lethally irradiated mice transplanted with normal bone marrow, there was no relation between the number of MK colonies and the macroscopic colony count at 10 days. When donor mice were injected with 5-FU 1 day before transplantation there was only a relatively small decrease in the marrow content of MK CFU-S, although the content of CFU-S10 was markedly decreased. Seven days after 5-FU injection, there was a pronounced increase in the ratio of both MK CFU-S and CFU-S10 to other nucleated cells in the marrow, but only in the case of MK CFU-S was the total number of cells increased above normal values. In addition, the velocity sedimentation pattern of MK CFU-S in FU-7 bone marrow was distinct from that of CFU-S10. It thus appears that MK CFU-S and CFU-S10 are separate entities.

In the present study, MK colonies were scored regardless of whether they were found isolated or within a colony containing other cell types. “Mixed” colonies were encountered in spleens seeded with FU-7 marrow, when CFU-S10 showed evidence of recovery, but were not observed after FU-1 marrow. Early investigations showed that most colonies present in the spleen 10 days after transplantation of normal marrow
are of a single cell type. The "mixed" colonies that are found could be due to pluripotent CFU-S,0 cells, but may also arise if seeding results in juxtaposition of progenitor cells of different types. More recent work has shown the importance of the assay interval with regard to types of colonies observed. Pluripotential cells produce colonies that become macroscopic at 11 days or later, while colonies that are larger at earlier times are transient and predominantly erythroid.

The modal sedimentation velocity of MK CFU-S from normal marrow (4.16 ± 0.05 mm/hr) is comparable to that obtained by others for MK colony-forming cells in vitro (MK-CFC). The shift in modal sedimentation velocity of MK CFU-S observed in FU-7 marrow (4.86 ± 0.16 mm/hr) is consistent with the movement of a predominantly resting cell population into cell cycle following perturbation with 5-FU. However, the reason for the bimodal MK CFU-S velocity sedimentation profile in FU-1 marrow is unclear and warrants further investigation. It is possible that two discrete subclasses of MK CFU-S with differing cycling characteristics are present. Such an explanation has been proposed to account for differences in sensitivity of MK progenitor cells to S-phase-specific drugs in vivo and in vitro.

In addition to displaying different velocity sedimentation characteristics to those from normal marrow, MK CFU-S that survive exposure to 5-FU in the donor for 24 hr before transplantation generate a higher average number of MK per colony. These results could be explained if the population of MK CFU-S is normally heterogeneous with respect to proliferation capacity and that there exist more "primitive" MK CFU-S of greater than average proliferative potential which are not affected by 5-FU because they are quiescent. In the 24 hr following the drug, such cells might be stimulated as a consequence of the 5-FU-induced perturbation, in a manner similar to the stimulation of quiescent salivary gland acinar cells following isoprenaline injection. On transplantation these stimulated MK CFU-S would enter into cycle earlier and undergo more divisions in the spleen than usual in the 10-day period before sampling. Left in donor mice for 7 days following 5-FU injection, the more primitive MK CFU-S would give rise to larger numbers of more mature cells, which would produce colonies containing fewer MK after transplantation.

MK CFU-S present 1 day after 5-FU behave like high proliferative potential granulocyte/macrophage-colony-forming cells, in that they have a high proliferative capacity and are activated as a consequence of the death of more mature proliferating cells caused by the drug. Primitive MK CFU-S are not detected when normal marrow is transplanted, since it presumably takes more than 10 days for them to produce MK in the spleen of an irradiated host if transplanted in an unstimulated form. The relationship of MK CFU-S detected in FU-1 marrow to CFU-S,0 in the same marrow, that is, cells that give rise to spleen colonies at day 13 and that are less affected by 5-FU than CFU-S,0, is not known at present. It could be that the former are progeny of the latter, and that MK are the first differentiated product that can be detected arising from CFU-S,0. On the other hand, these MK CFU-S may be primitive committed progenitors of MK. The survival of primitive MK CFU-S after 5-FU injection could account for the increased megakaryocytopoiesis seen 7–8 days later in the marrow of the drug-treated animal.

The relationship of MK CFU-S and MK CFC remains to be determined. Heterogeneity has been found in both colony size and cell ploidy in studies of megakaryocyte colony development from marrow cells in vitro, implying heterogeneity among MK CFC. It has been reported additionally that megakaryocyte colonies in agar culture can be classified into two types, one containing only large cells, while the other also includes small cells. We did not observe this in the present study; however, it would be difficult to detect the small cells with certainty in histologic sections in view of their low ploidy and the dispersed nature of megakaryocytes in colonies in the spleen. One difference that has emerged between primitive MK CFU-S and MK CFC that give rise to colonies in agar culture is that the latter are markedly decreased after 5-FU injection (J. Levin, personal communication). Using plasma clot cultures, Paulus et al. found they could detect three megakaryocyte progenitor compartments, with different probabilities of the cells becoming polyploid at the next division. Those progenitors with the greatest proliferative potential were found in mixed megakaryocytic/erythroid colonies. The relation between these progenitors and the primitive MK CFU-S that survive 5-FU is, at present, not known, as the effects of 5-FU on these cells have not been studied.

The data on MK CFU-S is consistent with an age-structured model of cell production in which cells with the highest proliferative potential cycle slowly and the more mature cells of the lineage with a lower proliferative potential cycle rapidly.

REFERENCES


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