Increases in Circulating Megakaryocyte Growth-Promoting Activity in the Plasma of Rats Following Whole Body Irradiation

By Masayoshi Miura, Carl W. Jackson, and Shirley A. Lyles

To gain insight into the regulation of megakaryocyte precursors in vivo, we assayed (in vitro) megakaryocyte growth-promoting activity (Meg-GPA) in plasma of rats in which both marrow hypoplasia and thrombocytopenia had been induced by irradiation. Rats received whole body irradiation of 234 rad from a $^{137}$Cs source. Plasma was collected at intervals of hours to days, up through day 21 postirradiation, and was tested, at a concentration of 30%, for Meg-GPA on bone marrow cells cultured in 1.1% methylcellulose with $5 \times 10^{-8}$ M 2-mercaptoethanol. With normal rat plasma, no megakaryocyte colonies (defined as $>4$ megakaryocytes) were seen and only a few single megakaryocytes and clusters (defined as 2 or 3 megakaryocytes) were formed. Two peaks of plasma Meg-GPA were observed after irradiation. The first appeared at 12 hr, before any decrease in marrow megakaryocyte concentration or platelet count. The second occurred on days 10–14 after irradiation, after the nadir in megakaryocyte concentration and while platelet counts were at their lowest levels. A dose–response study of plasma concentration and megakaryocyte growth, using plasma collected 11 days postirradiation, demonstrated that patterns of megakaryocyte growth were related to plasma concentration; formation of single megakaryocytes was optimal over a range of 20%–30% plasma concentration, while cluster and colony formation were optimal at a plasma concentration of 30%. All forms of megakaryocyte growth were decreased with 40% plasma. There was a linear relationship between the number of bone marrow cells plated and growth of single cells, clusters, and colonies using a concentration of 30% plasma collected 11 days after irradiation. We conclude that irradiation causes time-related increases in circulating megakaryocyte growth-promoting activity. We suggest that the irradiated rat is a good model for studying the relationships between Meg-GPA and megakaryocyte and platelet concentration in vivo.

STUDIES OF MURINE and human megakaryocytopoiesis in vitro have increased our understanding of the regulation of thrombopoiesis. Megakaryocyte colony formation has been obtained with media conditioned by spleen cells incubated with 2-mercaptoethanol or pokeweed mitogen and cell lines (WEHI-3, Buffalo rat liver-3A). The role of these stimulators in the physiologic regulation of megakaryocytopoiesis, however, has not been defined. Recently, Hoffman et al. reported that serum from patients with aplastic anemia or amegakaryocytic thrombocytopenia significantly enhanced the plating efficiency and size of human megakaryocyte colonies, but serum from patients with idiopathic thrombocytopenic purpura was not more conducive to growth than that from normals. They suggested that the stimulatory activity in the serum of aplastic anemic and amegakaryocytic thrombocytopenic patients was related to decreased megakaryocyte mass rather than low platelet counts. In contrast, Enomoto et al. and Kawakita et al. reported that urine extracts, not only from patients with aplastic anemia but also from ones with idiopathic thrombocytopenic purpura, who usually possess a normal or increased megakaryocyte mass, stimulated both in vitro and in vivo increases in megakaryocyte and platelet concentration in rodents.

To address some of the questions raised by these studies, an experimental model was needed in which the thrombopoietic system could be suppressed to produce marrow hypoplasia that was similar to that in these clinical situations. One treatment that reproducibly results in marrow hypoplasia and thrombocytopenia is sublethal irradiation. In this study, we have assayed (in vitro) the megakaryocyte growth-promoting activity (Meg-GPA) of plasma collected at intervals after sublethal irradiation of rats to determine whether this activity increases as megakaryocytes and platelets decrease postirradiation. Our experiments indicated that this is a good model for studying the relationships between Meg-GPA and megakaryocyte and platelet concentration in vivo. In this report, we have used the term “growth-promoting activity” rather than “colony-stimulating activity” because the plasmas studied promoted the formation of single megakaryocytes as well as clusters of 2 or 3 and colonies of 4 or more megakaryocytes.

MATERIALS AND METHODS

Animals

Male rats of the Long-Evans hooded strain, weighing 250–349 g, were purchased from Blue Spruce Farms (Altamont, NY).

Irradiation of Rats

Rats, placed in plastic irradiation holders (4–6 animals at a time) and rotated in the radiation field, were given 834 rad of whole body
radiation at a dose rate of 417 rad/min from a cesium 137 irradiator. After irradiation, the rats were housed in cages connected with automatic watering equipment and were supplied with circulating filtered, UV-irradiated water to help prevent infectious disease.

**Collection of Blood and Marrow Samples**

Blood for peripheral counts was obtained from etherized rats by pricking a Vaseline-coated lateral leg vein with a 27-gauge needle. Ten microliters of blood for platelet count was diluted in a Unopette reservoir containing 0.99 ml of 1% ammonium oxalate. Platelets were counted by phase microscopy.

Blood for hematocrit determination was collected in heparinized 75-mm capillary tubes (Sherwood Medical Industries, St. Louis, MO). Blood for plasma to be tested for Meg-GPA was drawn from the dorsal aorta of rats under ether anesthesia into syringes containing 300 U of preservative-free heparin. Plasma from 3 or 4 rats was combined at each time and stored at −80°C until used for assay for Meg-GPA. Serum was not used in this study because Messner et al. reported better human megakaryocyte colony growth using plasma. In addition, Vainchenker et al. reported that human serum contains a factor(s) capable of inhibiting megakaryocyte colony formation.

**Bone Marrow Megakaryocyte Concentration**

The average number of megakaryocytes per high power field (500× magnification) was determined on sternal bone marrow sections that were fixed in B-5 fixative, decalcified, and stained with hematoxylin and eosin. One entire longitudinal section was evaluated for each rat, and values were expressed as percent of untreated controls (control = 100%). Megakaryocyte concentration was not corrected for changes in megakaryocyte size, as we felt that the raw megakaryocyte concentration was a better reflection of megakaryocyte mass, which was of more interest in this study.

**Bone Marrow Megakaryocyte Diameter**

Megakaryocyte diameter was measured using an eyepiece micrometer. The diameter was expressed as the square root of the product of two measurements made at right angles. In most cases, 50 megakaryocytes in sternal marrow sections from each of three or four irradiated and four untreated control rats were measured at a magnification of 1,250×.

**Cell Preparation for Marrow Culture**

Tibial marrow was collected immediately after etherized rats were killed by exsanguination. The marrow was suspended in 3 ml of alpha media by gentle pipetting, passed through a 25-gauge needle by Messner et al. was used, except that conditioned media was added on top of the cultures, and they were allowed to stain 30–40 min at room temperature. This modification was necessary to prevent stain precipitation in the dishes. Because the megakaryocytes are the only hematopoietic cells of the rat that contain histochemically detectable acetylcholinesterase activity, mega-karyocyte colonies were defined as four or more tightly grouped acetylcholinesterase-positive cells. Single megakaryocytes and clusters of 2 or 3 were also enumerated. This classification was used so that all megakaryocyte growth could be evaluated. We distinguished between colonies and clusters so that our results could be more easily compared with those of others using different definitions for megakaryocyte colonies.

**RESULTS**

**Effect of Irradiation on Bone Marrow Megakaryocyte Concentration and Blood Platelet Count**

Figure 1 shows changes in megakaryocyte and platelet concentrations during the first 21 days after whole body irradiation of 834 rad. On day 1, the concentration of megakaryocytes in sternal marrow and platelets in blood increased above the control levels, in agreement with previous reports. Megakaryocyte concentration then declined to about one-half of baseline by day 3 and reached a nadir of less than 10% at 7 days. The decline of the platelets lagged 2 days behind that of megakaryocytes. On day 10, megakaryocyte concentration began to increase; the peripheral platelet count increased correspondingly, with the increase lagging behind that of megakaryocytes by about 4 days.

**Bone Marrow Megakaryocyte Diameter**

Megakaryocyte diameter increased after irradiation. The cells reached a maximum diameter, which was about 30% greater than that of untreated controls, by day 8, and remained near that level for the remain-
Hematocrit Levels After Irradiation

Hematocrit levels at various times after 834 rad of irradiation are shown in Fig. 3. They rose slightly during the first 5 days to a peak of 48%. After day 5, the hematocrit decreased to a low of 28% on day 12 after irradiation. By day 21, the hematocrit (40%) had recovered almost to the normal level.

In Vitro Megakaryocyte Growth-Promoting Activity (Meg-GPA) of Plasma After Irradiation

No megakaryocyte colonies were seen in cultures containing normal rat plasma, whereas a few single megakaryocytes and clusters of 2 or 3 megakaryocytes were observed. Colonies formed in the presence of plasma from irradiated rats were tight and compact, and the megakaryocytes were usually recognizable in unstained cultures by their large size, rather angular shape, translucent cytoplasm, and refractory cell border (Fig. 4). Two peaks of Meg-GPA were observed. The first increase occurred very early, at 12 hr after irradiation (Fig. 5), which preceded the increase in bone marrow megakaryocyte concentration at day 1. Plasma collected at 12 hr also stimulated the formation of granulocyte-macrophage (CFU-GM) and dimethoxybenzidine (DMB) positive colonies (data not shown), confirming a previous report for the former. After 12 hr, Meg-GPA decreased rapidly to baseline at 24 hr. This study of the early effects of irradiation on Meg-GPA has been repeated twice with similar results, i.e., a substantial increase in Meg-GPA was consistently found, with a peak at 12 hr after irradiation. The number of single cells [18.0 ± 3.2

Fig. 2. Megakaryocyte diameter after irradiation (834 rad). Each point represents the mean ± SEM for cells from 3 or 4 rats; values are expressed as percentage of untreated controls. The control megakaryocyte diameter was 20 ± 0.5 μm (4 rats).

Fig. 3. Hematocrit of rats after irradiation (834 rad). Each point represents the mean ± SEM for 3 or 4 rats.

Fig. 4. Example of a rat megakaryocyte colony after culturing for 7 days. (A) Unstained colony (note refractory cell border and rather angular shape); (B) same colony as A, showing positive reaction after 30-min incubation with modified acetylcholinesterase substrate added directly to the dish.

Fig. 5. Megakaryocyte growth-promoting activity of plasma collected early after irradiation (834 rad). Open circles, colonies of 4 or more megakaryocytes; closed triangles, clusters of 2 or 3 megakaryocytes; closed circles, single megakaryocytes. Each point represents the mean ± SEM for 4 replicate dishes.
Meg-GPA IN PLASMA FROM IRRADIATED RATS

(SEM) per 2 x 10^5 cells plated] induced by normal plasma in the experiment shown was much higher than usual. In 8 other experiments, for which data are given in this report, the average number of single megakaryocytes induced by normal plasma ranged from 2.5 to 7.0 (mean of 4.3 ± 0.6). The reason for this higher value is not known.

The second peak of Meg-GPA, which appeared at 10–14 days (Fig. 5), coincided with the nadir in platelet count, while megakaryocyte recovery had already begun. Thereafter, megakaryocyte colony-stimulating activity decreased to near baseline on day 21 after irradiation. Formation of single megakaryocytes and clusters was more frequent than colony formation but followed a similar pattern except that stimulating activity for single cells and clusters remained elevated at day 21. At the time of this particular experiment, single megakaryocytes and clusters were not distinguished from each other during counting, and thus, the data in Fig. 6 reflect the combination of these two types of growth. As seen in Figs. 5, 7, and 8, single cells accounted for 70%–77% of these events. The ratio of the combined single and cluster events to colonies during the 10–14-day postirradiation period was slightly more than 2:1; however, at earlier (Fig. 5) and later times (after 15 days), this ratio was much higher. These assays of growth-promoting activity have been repeated with similar results.

Relationship Between Megakaryocyte Growth In Vitro and Plasma Concentration

The dose–response relationship between growth of megakaryocytes in vitro (evaluated at 7 days) and the percentage of rat plasma (collected 11 days after irradiation) in the culture is shown in Fig. 7. Ten percent plasma from irradiated rats stimulated formation of single megakaryocytes to a level approximately two-thirds of that stimulated by the optimal plasma concentration (30%). Although growth with 5% plasma was not examined in this experiment, in a separate dose–response assay, cultures containing 5% plasma had 1.5 ± 0.5 (SEM) single cells, 1.0 ± 0.6 clusters, and no colonies, whereas 10% plasma produced 19.5 ± 1.7 single cells, the same as seen in the experiment in Fig. 7. Clusters of 2 or 3 megakaryocytes and colonies of 4 or more megakaryocytes were most numerous with 30% plasma. All forms of megakaryocyte growth were decreased with 40% plasma.
**Relationship Between Number of Marrow Cells Cultured and Quantity of Megakaryocyte Growth**

Using a concentration of 30% plasma collected 11 days after irradiation, there was a linear relationship passing through zero between the number of bone marrow cells plated and the growth pattern of megakaryocytes in vitro (Fig. 8). The relationship between cells plated and colonies formed showed a correlation coefficient (r) of 0.944 (p < 0.02). The regression line estimated by the least squares method for colonies had a slope of 5.19, with an intercept of 0.14. The r value (0.954, p < 0.02) and regression line (slope = 4.62, intercept = 1.10) for clusters were quite similar to those of colonies. In contrast, the slope (10.80) for single cells was twice that of colonies. In contrast, the slope (10.80) for single cells was twice that of colonies and clusters, whereas the intercept (1.59) was similar. The r value for single cells was 0.986 (p < 0.005).

**Effect of 2-Mercaptoethanol on Megakaryocyte Formation In Vitro**

The effect of 2-mercaptoethanol was determined by comparing megakaryocyte formation in cultures with or without the compound. Cultures contained 30% plasma collected from rats 11 days postirradiation and were evaluated on day 7. Fewer (about one-half as many) megakaryocyte single cells, clusters, and colonies were observed in the dishes without 2-mercaptoethanol in 3 of 4 experiments. The results of a representative experiment are shown in Table 1.

**DISCUSSION**

Megakaryocyte colony formation has been studied in cultures containing conditioned media produced in vitro, but little is understood about the physiologic regulation in vivo of the hematopoietic precursors that form these colonies and the relation of these stimulatory factors to megakaryocyte and platelet number. Therefore, we have studied Meg-GPA using plasma collected from rats at various times after irradiation. Two peaks of circulating Meg-GPA were observed. The first appeared at 12 hr after irradiation, before any decrease in marrow megakaryocyte concentration or platelet count. The mechanism involved in this response is unknown; however, a recent report suggested that the addition of irradiated bone marrow cells to cultures enhanced the formation of secondary mixed hematopoietic colonies may be related. Perhaps growth-promoting factors were being released from damaged cells when the marrow cellularity began to decrease 12 hr after irradiation. It is known, for example, that plasma from x-irradiated rats contains factors that induce leukocytosis in vivo.

The second Meg-GPA peak at 10–14 days postirradiation probably was related to the decreased megakaryocyte and platelet concentrations at that time. From patient studies, Hoffman et al. suggested that megakaryocyte colony-stimulating activity was related to megakaryocyte concentration rather than to platelet number. In our study, however, the highest Meg-GPA coincided more closely with the period of lowest platelet counts than with decreased megakaryocyte number. This question has not been resolved, as conflicting results have been reported from different laboratories using plasma or serum of animals made acutely thrombocytopenic with platelet antiserum. Levin et al. found that plasma from rabbits made acutely thrombocytopenic by injection of platelet-specific antiserum did not stimulate formation of megakaryocyte colonies in vitro. However, Nakeff reported that serum from mice made thrombocytopenic with antiplatelet serum did induce megakaryocyte colony formation. Levin et al. pointed out that injection of antiplatelet serum may affect other hematopoietic cells and that the response may be more complex than originally thought. More definitive experiments will be required to settle the issue of whether Meg-GPA is related to megakaryocyte or platelet number.

It is well known that changes in blood and plasma volume occur following irradiation and that a slightly increased hematocrit level during the first 5 days after irradiation does not necessarily reflect actual changes in the total number of red cells in the bloodstream. Later decreases of hematocrits are due mainly to decreased erythropoiesis and hemorrhage. Although it has been reported that megakaryocyte culture systems require erythropoietin for the maximum development of colony growth, no clear relationship was observed between hematocrit levels of rats and Meg-GPA on a day-to-day basis.

As part of this study, we determined some characteristics of megakaryocyte growth in culture using 11-day postirradiation plasma as a stimulator. In addition to studying colony formation, we examined the formation of single megakaryocytes and clusters of 2

**Table 1. Effect of 2-Mercaptoethanol on Megakaryocyte Formation in Cell Culture**

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<tr>
<th>2-Mercaptoethanol*</th>
<th>Megakaryocyte Growth from 2 × 10⁶ Marrow Cells†</th>
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<tr>
<td>+</td>
<td>Single: 27.5 ± 2.6†</td>
<td>Cluster: 14.5 ± 2.2</td>
<td>Colony: 12.0 ± 0.8</td>
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<td>-</td>
<td>16.5 ± 0.5</td>
<td>8.5 ± 1.5</td>
<td>5.5 ± 1.0</td>
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*5 × 10⁻⁴ M.†Cultures contained 30% plasma collected from rats 11 days postirradiation.†Mean ± SEM.†Cultures contained 30% plasma collected from untreated rats and 5 × 10⁻⁴ M 2-mercaptoethanol contained 7.0 ± 0.6 single megakaryocytes, 2.5 ± 1.9 megakaryocyte clusters, and no megakaryocyte colonies.
or 3. Single megakaryocytes formed in this system, as has been previously reported by others using different in vitro conditions. Using 30% plasma from irradiated rats as stimulus, the dishes had 18.5–54.0 (median 32.7; 8 experiments) single megakaryocytes per 2 x 10^7 bone marrow cells. These results indicate that single megakaryocytes differentiate from precursors in vitro with the stimulus of plasma from irradiated rats. The precursors of single megakaryocytes may be more sensitive to the stimulus than precursors of colony-forming cells for the following reasons. (1) Stimulating activity for single megakaryocytes and clusters appeared earlier after irradiation and remained later than that for megakaryocyte colonies. (2) Ten percent plasma from irradiated rats stimulated about two-thirds the number of single megakaryocytes, as did the optimal plasma concentration (30%), but 10% plasma was not effective in stimulating colony formation. (3) It has been reported that the proportion of acetylcholinesterase-positive cells in vivo increases soon after induction of acute thrombocytopenia, whereas there is no change in the number of megakaryocyte colony-forming cells in vitro until several days after induction of acute thrombocytopenia. Williams et al. postulated that two factors are required for megakaryocyte colony formation. One factor, megakaryocyte colony-stimulating factor (Meg-CSF), stimulates cell division and, therefore, colony formation. The other, termed megakaryocyte potentiator, would induce differentiation, increasing the size and ploidy of the cells within a colony. The requirement of two factors for colony formation might explain why the ratio of single cells and clusters to the colonies induced by plasma collected earlier or later was considerably higher. Megakaryocyte potentiator, which may induce differentiation of single cells, might be present earlier and later in the experiment, whereas Meg-CSF might be produced in the rats during the second week postirradiation. Alternatively, the plasma from irradiated rats may contain only one factor that induces megakaryocyte growth in vitro, but the amount required for colony formation may be greater than that necessary for single cell and cluster formation.

Different characteristics of megakaryocyte colonies described in reports from various laboratories make comparisons of results difficult. As the recent review of Levin indicated, some confusion is undoubtedly due to the complexity of current culture systems and the uncharacterized components that have been used. Besides the protein compounds, such as fetal calf, horse, or human serum, a successful culture system also needs additional stimuli, such as conditioned media and erythropoietin, as well as serum and urine extracts from patients with thrombocytopenia. In particular, variations in conditioned media used by different investigators have hampered progress. The use of plasma from irradiated rats makes the in vitro megakaryocyte culture system simpler and more reproducible. Using plasma collected 11 days postirradiation, when there is a linear relationship passing through zero between the number of marrow cells plated and number of colonies, clusters, and single cells formed, provides an assay that can be used quantitatively. 2-Mercaptoethanol seems to be essential to optimize the megakaryocyte growth in this system.

In summary, the irradiated rat is not only a reliable and reproducible source of Meg-GPA, but also a good model with which to study the relationship between Meg-GPA and megakaryocyte or platelet concentration in vivo. Plasma from irradiated rats should also be useful as a stimulus to establish the hierarchy in megakaryocyte differentiation of single megakaryocytes and colonies that form in vitro.

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