Effect of Recombinant Granulocyte-Macrophage Colony-Stimulating Factor on Murine Thrombocytopoiesis In Vitro and In Vivo

By Toshiyuki Ishibashi, Hideo Kimura, Yayoi Shikama, Tatsumi Uchida, Shigeo Kariyone, and Yukio Maruyama

To investigate the effect of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) on murine megakaryocytopoiesis in vitro, the factor was added to both serum-free colony assays and liquid marrow cultures. GM-CSF had a significant megakaryocytic colony-stimulating activity. After 2 hours of preincubation with and without 10 ng/mL rGM-CSF, the percentage of megakaryocyte colony-forming cell (CFU-MK) in DNA synthesis was determined by tritiated-thymidine suicide using colony growth. The reduction of CFU-MK colony numbers in marrow culture was 47.5% ± 9.9%, 20.9% ± 5.2% (control), respectively, indicating that the factor affected cell cycle at CFU-MK levels. When acetylcholinesterase (AchE) production was measured fluorometrically after 4 days of liquid culture, rGM-CSF elicited an increase in AchE activity in a dose-dependent fashion. To determine if the hematopoietin acts directly on megakaryocytic differentiation, 2 ng/mL rGM-CSF was added to serum-free cultures of 295 single megakaryocytes isolated from CFU-MK colonies.

Granulocyte-Macrophage colony-stimulating factor (GM-CSF), which regulates or modulates the proliferation and differentiation of granulocyte, macrophages, and several other hematopoietic progenitors, has been purified to homogeneity and molecularly cloned. Several investigators have reported that recombinant GM-CSF (rGM-CSF) stimulates megakaryocytic colony formation in vitro in both humans and mice. However, the mechanism of this effect is unclear. Although treatment with rGM-CSF has been shown to be effective on granulocytopoiesis in vivo, the influence of this factor on platelet production is unknown.

In this study, we further characterized the in vitro effect of rGM-CSF by determining if GM-CSF acts directly on murine megakaryocytopoiesis. In addition, we assessed the effect of in vivo administration of rGM-CSF on the platelet production of mice.

MATERIALS AND METHODS

Marrow cell preparation. Six- to eight-week-old specific pathogen-free C57B1/6 male mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were killed by cervical dislocation. Bone marrow from the femur was flushed and suspended into Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY). A mononuclear suspension was prepared by repetitive centrifugation at 250 × g for 10 minutes at room temperature and resuspended in IMDM.

Serum-free liquid marrow culture. Whole marrow cells (1 × 10^6 per well) were cultured in a final volume of 0.2 mL of IMDM containing 1% Nutricyte (a serum-free medium supplement; I. Brooks Laboratory, San Diego, CA), 0.05% crystalized bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO) and 100 U/mL of penicillin-streptomycin (PS) in 96 microwell plates as previously described. Various concentrations of recombinant mouse GM-CSF4 (specific activity 5 × 10^10 U/mg; a gift of Sumitomo Pharmaceutical Inc, Osaka, Japan) were added to marrow cultures. Units of activity were defined by granulocyte-macrophage colony-forming cell (CFU-GM) colony formation in mouse bone marrow culture. Half-maximal colony formation was assigned to 50 U/mL.

Tritiated thymidine (3HTdR) incorporation into serum-free liquid cultures. The effect of GM-CSF on cell proliferation was determined by 3HTdR incorporation. Marrow cells were cultured in agar (1.065/1.077 g/cm^3 discontinuous Percoll gradient as previously described). After 2 days of culture, 0.5 μCi of 3HTdR (methyl-3H thymidine, 25 Ci/mmol, Amersham International, Buckinghamshire, England) was added to each well. Sixteen hours later, the cells were harvested with an automatic multiple cell harvester (Labo Mash; Labo Science Co, Ltd, Tokyo, Japan) onto glass fiber paper, and the radioactivity measured with a scintillation counter.

Serum-free colony assays. To assess the influence of rGM-CSF on the detection of megakaryocytic (CFU-MK) and granulocyte-macrophage (CFU-GM) colony-forming cells, marrow cells were cultured at 1 × 10^6 cells/mL in agar in 1-mL final volumes in 35-mm culture dishes as previously described, with the exception that serum was replaced with 1% bovine serum albumin (BSA), 360 μg/mL human transferrin, and 0.98 μg/mL cholesterol. GM-CSF and heated material (100°C, 40 minutes) were added to each well at concentrations ranging from 0.05 to 10 ng/mL. After 7 days of culture, colonies were scored.

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culture, each dish was fixed with 2% glutaraldehyde onto glass slides. Acetylcholinesterase (AchE) staining was performed to detect CFU-MK colonies, followed by hematoxylin staining for CFU-GM colonies. Three to four replicate plates were counted for each experiment.

**Progenitor cell cycle analysis.** To examine the effect of rGM-CSF on the DNA synthetic phase (S phase) of progenitors, HTdR suicide studies were performed as previously described. Briefly, nonadherent marrow cells were preincubated with 10 ng/mL rGM-CSF at 37°C for 2 hours, washed three times, and exposed to 1HTdR (25 Ci/mmol, Amersham International) at 37°C for 20 minutes. Control cells were exposed to 1.2 μg/mL of cold thymidine. The cells were washed three times and cultured in colony assays using 7.5% pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) as a colony-stimulating activity.

**Quantitation of AchE activity.** AchE, a relatively specific marker of the megakaryocytic lineage in mice, was measured fluorometrically to quantify proliferation and differentiation of murine megakaryocytosis in liquid cultures as previously described. Briefly, after 4 days of culture, the plate was centrifuged and the supernatant was discarded. Two-tenths milliliter of a solution of 0.2% Triton X-100 (Sigma) in 1 mmol/L EDTA (Sigma), 0.12 mol/L NaCl, and 50 mmol/L HEPES pH 7.5 (Flow Laboratories, Irvine, UK) was added to each well followed by the addition of 20 μL of acetylthiocholine iodide (final concentration 0.56 mmol/L). After 4 hours of incubation, 10 μL of 0.4% coumarin phenylmaleimide (Molecular Probes Inc, Junction City, OR) was added to 10 μL of the reaction mixture, followed by 2 mL of diluent buffer containing 0.2% Triton X-100 in 1 mmol/L EDTA and 5 mmol/L sodium acetate pH 5.0. The fluorescence emission was measured with a filter fluorometer with an excitation filter of 390 nm and an emission filter of 450 nm.

**Single cell culture of CFU-MK-derived megakaryocytes.** Single megakaryocytes isolated from CFU-MK colonies were cultured as previously described. Briefly, bone marrow was enriched for progenitor cells on a 1.065/1.077 g/cm³ discontinuous Percoll gradient. Twenty-five thousand cells were cultured with various concentrations of rGM-CSF in serumless liquid cultures. Data are expressed as the mean ± SD of three experiments. The radioactivity of four replicate wells was measured for each experiment.

**Histologic assessment of bone marrow megakaryocytes.** Bone marrow samples of the femurs were fixed with 10% formalin to determine the in vivo effect of GM-CSF on megakaryocytes. The samples were dehydrated and blocked in paraffin. Megakaryocyte measurement was performed on 3-μm sections and the number and size were determined as previously described. Statistical analysis. Statistical analyses were performed by Student’s t test with the exception of chi-square analysis for single cell study.

## RESULTS

**Marrow cell proliferation.** The influence of rGM-CSF on cellular proliferation was examined by HTdR incorporation assay in serumless liquid culture. As shown in Fig 1, a significant increase in radioactivity was observed at 0.05 ng/mL of rGM-CSF (P < .01), which appears more likely to

### Table 1. Effect of rGM-CSF on Colony Growth

<table>
<thead>
<tr>
<th>GM-CSF (ng/mL)</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-MK colonies (1 x 10⁵ cells)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 ± 1</td>
<td>4 ± 2</td>
<td>7 ± 2</td>
<td>15 ± 2</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>CFU-GM colonies (1 x 10⁵ cells)</td>
<td>0</td>
<td>5 ± 1</td>
<td>31 ± 6</td>
<td>95 ± 14</td>
<td>141 ± 28</td>
<td>193 ± 12</td>
<td>203 ± 10</td>
<td>212 ± 11</td>
<td>221 ± 14</td>
</tr>
</tbody>
</table>

After 7 days of culture, CFU-MK colonies were detected by AchE staining, followed by hematoxylin staining for CFU-GM colonies. Results are expressed as the mean ± SD per 10⁵ cells of five experiments. Three to four replicated plates were enumerated for each experiment. One nanogram per milliliter of GM-CSF was required to support CFU-MK colony growth (P < .01).
To determine the effect of rGM-CSF on the frequency of the DNA synthetic phase in progenitor cells, 10 ng/mL of rGM-CSF was added to nonadherent marrow cells. After 2 hours of incubation at 37°C, ²HrTdR suicide assay was performed as previously described. An apparent increase in the percentage of CFU-MK and CFU-GM in S phase was induced by the treatment of rGM-CSF (P < .01).

*Marrow cells were preincubated without rGM-CSF.

reflect the proliferation of granulocyte-macrophage lineage than megakaryocytic proliferation. Plateau incorporation induction occurred at an rGM-CSF dose of 2 ng/mL.

Colony assays. Table 1 shows the effect of rGM-CSF on progenitor cells. GM-CSF induced megakaryocytic colony formation at a concentration of 1 ng/mL (P < .01) with further stimulation at higher concentrations, while CFU-GM colony formation was stimulated by the factor in a dose-dependent fashion at concentrations greater than 0.05 ng/mL (P < .01). This observation was accompanied by an increase in cell number and size in individual CFU-MK colonies (data not shown). A heated GM-CSF did not support either CFU-GM or CFU-MK colony growth at any concentrations added.

Influence of rGM-CSF on the S phase of progenitors. As shown in Table 2, 2 hours of preincubation with 10 ng/mL of rGM-CSF induced CFU-MK into cell cycle, with 47% ± 10% of CFU-MK in S phase compared with 21% ± 5% in the absence of the factor (P < .01). As would be expected, the treatment with rGM-CSF increased the percentage of CFU-GM in S phase with 46% ± 6%, compared with 22% ± 4% in the absence of the factor (P < .01).

Effect of rGM-CSF on AchE activity in liquid marrow culture. AchE activity was measured fluorometrically after 4 days in serumless liquid culture. Figure 2 shows a significant increase in AchE activity at a concentration of 0.05 ng/mL (P < .01), with further increments noted at higher concentrations. Figure 3 demonstrates AchE staining for murine megakaryocytes in 96 well plates. The increase in the size and apparent number of megakaryocytes was observed in rGM-CSF-stimulated when compared with control cultures.

Single cell culture. To determine if rGM-CSF acted directly on megakaryocytic differentiation, 2 ng/mL of this factor was added to single megakaryocytes isolated from 5-day-old CFU-MK colonies. Cell diameter was measured on 295 megakaryocytes before and after culture. As shown in Table 3, an increase in size was observed in presence of rGM-CSF in 65% of cells initially 10 to 20 μm in diameter, 71% of cells 20 to 30 μm, and 40% of cells greater than 30 μm in diameter.
Table 3. Effect of rGM-CSF on the Size of Single Megakaryocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Megakaryocyte No.</th>
<th>Megakaryocyte Counts (×10^2/mm²)</th>
<th>Megakaryocyte Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (n = 6)†</td>
<td>83.90 ± 11.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 pg (n = 6)†</td>
<td>86.52 ± 10.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 pg (n = 3)†</td>
<td>81.99 ± 1.76</td>
</tr>
</tbody>
</table>

*One and five micrograms of rGM-CSF were administered intraperitoneally (ip) twice a day. Total 12 and 60 μg of rGM-CSF were injected over 6 consecutive days.

In vivo studies. Table 4 shows that rGM-CSF administration elicited about two- to threefold increase in absolute granulocyte numbers, although injections of a control buffer had negligible effects on granulocyte levels. No significant increase in platelet counts was observed in mice injected with concentrations of both 1 and 5 μg of rGM-CSF. When a control solution or 1 μg of heated rGM-CSF was injected, platelet and granulocyte levels did not change. Megakaryocyte number and size were determined on bone marrow sections in each specimen of 50 random megakaryocytes. As shown in Table 5, GM-CSF did not change either the number or the size in vivo in mice. Furthermore, neither CFU-MK or CFU-GM increased per 1 × 10^6 bone marrow cells after treatment of rGM-CSF. The total cellularity per femur decreased to 26.6 ± 3.0 × 10^6 cells from 30.2 ± 2.5 × 10^6 cells in mice injected with 1 μg of the factor (P < .05), while there was no significant change in 5-μg–injected mice. Approximately 60% elevation was observed in total spleen cells, whereas a 5.1-fold increase in nonerythroid progenitors per 1 × 10^6 cells was noted (Table 6). The apparent increase in CFU-MK and CFU-GM colony numbers from spleen after administration of 1 μg of GM-CSF was significant (P < .001).

DISCUSSION

Megakaryocytopoiesis has been postulated to be controlled by two factors:79 a megakaryocyte colony-stimulating activity29,30 and a maturational promoter, operationally termed thrombopoietin.79,31,32 Over the past decade, it has been shown that several known factors, including interleukin-3 (IL-3), erythropoietin (Ep), and GM-CSF have been involved in the regulation of megakaryocytopoiesis in vitro in humans and mice.6,31-33 We have demonstrated that IL-3 and Ep have an effect on mature megakaryocytes as well as megakaryocytic colony-forming cells,21,34 suggesting that these factors not only possess colony-stimulating activity but also maturational activity. In fact, Berridge et al35 have shown that administration of Ep increases the platelet counts in rats. More recently, Ganser et al36 have reported an increase in platelet numbers by IL-3 in vivo trial in humans.39 In addition, our laboratory has demonstrated that IL-6 induces a dramatic increase in platelets in vivo in mice.40

Several investigators have shown the stimulating activity of GM-CSF on megakaryocyte colony formation.6,41 However, neither the mechanism of its action or the effect on later stages of megakaryocytopoiesis have been studied. The colony data shown here was consistent with other reports38,39 regarding CFU-MK colony formation. To determine if rGM-CSF induced progenitor cells into cycle, the effect on the DNA synthetic phase was tested. Two hours of incubation with rGM-CSF increased the percentage of CFU-MK in S phase. These findings suggested that GM-CSF directly influenced the committed stem cells of the megakaryocytic lineage as well as the CFU-GM.

To determine if rGM-CSF promotes some aspects of megakaryocytic differentiation, AchE activity was measured in serumless liquid marrow culture. rGM-CSF increased AchE production at the same concentration (0.05 ng/mL) that the proliferation of granulocyte-macrophage lineage was primarily observed, with further increments at higher concentrations. However, this result does not preclude the possibility of mediation through accessory cells. Therefore, we measured the size of single megakaryocytes derived from CFU-MK colonies before and after culture under no interaction of other cells. An increase in size was observed in individual megakaryocytes with rGM-CSF when compared with control cells, indicating that rGM-CSF directly influenced the differentiated megakaryocytes.
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The effect of GM-CSF on granulopoiesis by studies in animals and clinical trials has been reported, the influence of the factor on platelet production has been variable. Nienhuis et al., Vadhan-Raj et al., and Antman et al. have shown some effect on platelet levels in primates and humans, while other investigators have not. Therefore, it is unlikely that the in vivo negative data might be due to the glycosylation of the growth factor.

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Second, GM-CSF in this study was nonglycosylated. Mayer et al. assessed the ability of two types of GM-CSF, one glycosylated and derived from CHO cells, and another nonglycosylated and extracted from Escherichia coli. Administration of both forms induced a granulocytosis of the same magnitude and duration in primates. Therefore, it is unlikely that the in vivo negative data might be due to the glycosylation of the growth factor.

Third, in vitro studies have shown that several factors have an effect on megakaryocyte proliferation and maturation. It is conceivable that GM-CSF alone might not be sufficient to promote the proliferation and differentiation of megakaryocytes and induce thrombocytosis. Administration of GM-CSF together with other hematopoietin(s) may be necessary to demonstrate any potential thrombopoietic capacity.

In conclusion, there is a disparity between the in vitro influence of GM-CSF on megakaryocytopoiesis and in vivo effect. Additional data will be required to determine if the lack of influence of the factor in vivo is merely technical or indeed represents a fundamental difference between the in vitro and in vivo situation.

ACKNOWLEDGMENT

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REFERENCES

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Table 6. Influence of rGM-CSF on the Bone Marrow and Spleen

<table>
<thead>
<tr>
<th>Group</th>
<th>Bone Marrow* (x10^6 cells)</th>
<th>Spleen+ (x10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU-MK</td>
<td>CFU-GM</td>
</tr>
<tr>
<td></td>
<td>(x10^6 cells)</td>
<td>(x10^6 cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µg‡</td>
<td>27 ± 4</td>
<td>122 ± 26</td>
</tr>
<tr>
<td>Control‡</td>
<td>28 ± 4</td>
<td>136 ± 31</td>
</tr>
<tr>
<td>5 µg‡</td>
<td>33 ± 4</td>
<td>118 ± 8</td>
</tr>
<tr>
<td>Control‡</td>
<td>32 ± 2</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>Control‡</td>
<td>31 ± 3</td>
<td>119 ± 2</td>
</tr>
</tbody>
</table>

*One microgram, n = 13; control, n = 12; 5 µg, n = 3; control, n = 3.
†One microgram, n = 6; control, n = 3; 5 µg, n = 3; control, n = 3.
‡One and five micrograms of rGM-CSF and a control solution were injected every 12 hours for 6 days.
Effect of recombinant granulocyte-macrophage colony-stimulating factor on murine thrombocytopoiesis in vitro and in vivo

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