In Vivo Effect of Human Granulocyte-Macrophage Colony-Stimulating Factor on Megakaryocytopoiesis

By Massimo Aglietta, Clara Monzeglio, Fiorella Sanavio, Franco Apra, Silvia Morelli, Alessandra Stacchini, Wanda Placibello, Federico Bussolino, GianPaolo Bagnara, Giorgio Zauli, Angelika C. Stern, and F. Gavosto

The effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on megakaryocytopoiesis and platelet production was investigated in patients with normal hematopoiesis. Three findings indicated that GM-CSF plays a role in megakaryocytopoiesis. During treatment with GM-CSF (recombinant mammalian, glycosylated; Sandoz/Schering-Plough, 5.5 μg protein/kg/d, subcutaneously for 3 days) the percentage of megakaryocyte progenitors (megakaryocyte colony forming unit [CFU-Mk]) in S phase (evaluated by the suicide technique with high ³H-Tdr doses) increased from 31% ± 16% to 88% ± 11%; and the maturation profile of megakaryocytes was modified, with a relative increase in more immature stage I-III forms. Moreover, by autoradiography (after incubation of marrow cells with ¹²⁵I-labeled GM-CSF) specific GM-CSF receptors were detectable on megakaryocytes. Nevertheless, the proliferative stimulus induced on the progenitors was not accompanied by enhanced platelet production (by contrast with the marked granulocytopoiesis). It may be suggested that other cytokines are involved in the regulation of the intermediate and terminal stages of megakaryocytopoiesis in vivo and that their intervention is an essential prerequisite to turn the GM-CSF-induced proliferative stimulus into enhanced platelet production.

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Materials and Methods

Patients. Patients with histologically proven neoplasia not involving the myeloid system and with normal bone marrow participated in the study. Each patient gave written informed consent, according to the Helsinki declaration. All patients had normal leukocyte counts and normal hemoglobin (Hb) levels. Liver and kidney functions were normal, and no signs of abnormalities were present. No concomitant treatments with corticosteroids, sulphonamides, H₂ antagonists, nonsteroid anti-inflammatory drugs, or lithium were administered.

GM-CSF. Recombinant GM-CSF (mammalian, glycosylated; Sandoz/Schering Plough) in purified lyophilized form was obtained from Sandoz Pharma Ltd, Basel, Switzerland.

Study design. GM-CSF was administered to six patients by subcutaneous route (2.8 μg of protein/kg every 12 hours for 3 days). Before and during the course of the study, the patients were monitored daily by recording of vital signs, physical examination, and determination of the complete blood counts with differential.

Bone marrow morphology. Megakaryocyte maturation was defined according to Williams and Levine.

Kinetic investigation of narrow-committed progenitors. The fraction of CFU-GM, BFU-E, and CFU-Mk in DNA synthesis (S phase) was assessed, as previously described, by the suicide technique after exposure to high-specific-activity tritiated thymidine (¹H-Tdr) before culturing in semisolid media.

The marrow light-density fraction was collected after Ficoll Hypaque (FH) separation (Lymphoprep, Nyegaard, Oslo, Norway), washed three times and resuspended in 0.5 mL of Iscove's modified Dulbecco's medium (IMDM; Flow Laboratories, Irvine, UK) + 10% fetal calf serum (FCS; Flow Laboratories) at a concentration of 2 x 10⁶ cells/mL. Five tubes were prepared and
incubated with medium (in duplicate), 0.5 mg/mL cold thymidine (Sigma Laboratories, St Louis, MO), \(^{3}H\)-Tdr (100 Ci/mmol, specific activity: 20 Ci/mmole/L; Amersham International, Buckinghamsire, UK, in duplicate). After 30 minutes of incubation at 37°C, the reaction was stopped by the addition of 5 mL ice-cold Hank's balance salt solution (HBSS; Flow Laboratories) containing 0.5 mg/mL unlabeled thymidine. After three washes, the cells were resuspended in IMDM: CFU-GM, BFU-E, and CFU-Mk cultures were prepared with previously described procedures\(^{8,9}\) by seeding 1 \(\times 10^{5}\) cells/dish (for CFU-GM and BFU-E) and 3 \(\times 10^{5}\) cells/dish (for CFU-Mk) of the original cell suspension. The following medium and growth factors were used: 10% conditioned medium of the 5637 cell line for CFU-GM; 10% human cord blood endothelium supernatant and 1.5 IU erythropoietin (Tyojobo, Osaka, Japan) for BFU-E; and 10% conditioned medium of the Mo cell line for CFU-Mk. After 7 and 14 days of incubation, the number of clones was evaluated by two independent investigators. CFU-GM and BFU-E were identified by their morphology, CFU-Mk identification was performed by means of J15 (CD41w), a monoclonal antibody (MoAb) directed against the glycoprotein (GP) IIb-IIIa complex: binding was shown by fluorescinated goat antimosue IgG (Ortho-Diagnostic System, Raritan, NJ).

The percentage of progenitors in the S phase of the cell cycle (Ns) was determined by applying the following formula: \(N_s = N_c - N_r/N_c\), where \(N_c\) is the number of colonies or bursts in the controls, and \(N_r\) is the number of colonies or bursts in the samples treated with high-dose \(^{3}H\)-Tdr.

Receptor studies. Marrow cells from normal donors were obtained with two separation techniques. In two cases (samples 1 and 2), buffy coat cells were obtained by centrifugation for 10 minutes at 800g. In one case (sample 3), enriched marrow megakaryocyte populations were obtained by separation on Percoll gradient (density 1.050).

Ten million cells per milliliter were incubated for 2 hours at room temperature in a rotating system, with 1,000 pmol/L \(^{125}\)I-GM-CSF with and without a 1,000-fold excess of unlabeled GM-CSF. They were then layered on a solution at 75% FCS. Cells were centrifuged for 10 minutes at 400g, suspended in phosphate-buffered saline (PBS), and cytospin slides were prepared. Slides were fixed for 30 seconds at room temperature in phosphate buffer with 8% formaldehyde and 65% acetone, covered with Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY), and incubated in the dark. After 4 weeks, slides were developed, fixed, and cells were counterstained with May-Grünwald-Giemsa. The degree of megakaryocyte labeling was evaluated under the light microscope by counting the number of grains per cell and subtracting the background labeling. Specific labeling is equal to the difference between the number of grains in specimens incubated with \(^{125}\)I-GM-CSF only and those incubated with unlabeled GM-CSF in excess.

### Table 1. Modifications of Peripheral Blood Parameters Occurring After Three Days of Treatment With GM-CSF

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Leukocytes (x10^9/L)</td>
<td>6.7 ± 1.5</td>
</tr>
<tr>
<td>Neutrophils (x10^9/L)</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>Eosinophils (x10^9/L)</td>
<td>0.16 ± 0.08</td>
</tr>
<tr>
<td>Monocytes (x10^9/L)</td>
<td>0.34 ± 0.20</td>
</tr>
<tr>
<td>Hb (g%)</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>Platelets (x10^12/L)</td>
<td>291 ± 49</td>
</tr>
</tbody>
</table>

Results are means ± SD of the values obtained from six patients. 
*P < .01 compared with data at day 0.

### Table 2. Effect of GM-CSF Treatment on the Percentage of S Phase Marrow Progenitors

<table>
<thead>
<tr>
<th>Day of Study</th>
<th>0</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7 CFU-GM</td>
<td>36 ± 8</td>
<td>59 ± 5t</td>
</tr>
<tr>
<td>Day 14 CFU-GM</td>
<td>40 ± 15</td>
<td>58 ± 6*</td>
</tr>
<tr>
<td>BFU-E</td>
<td>39 ± 14</td>
<td>71 ± 18t</td>
</tr>
<tr>
<td>CFU-Mk</td>
<td>31 ± 16</td>
<td>88 ± 14t</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD of the values obtained from six patients (CFU-Mk data refer to four patients).

### RESULTS

Subcutaneous administration of GM-CSF resulted in a marked granulo-monocytosis, whereas lymphocyte, erythrocyte, and platelet numbers were not affected (Table 1). These results are similar to those previously observed after continuous intravenous infusion in a similar group of patients.\(^6\)

Table 2 shows that the percentage of myelopoietic progenitors in S phase increased during treatment. A particularly interesting new finding was the increment in the proliferative activity of megakaryocyte progenitors (the percentage of S phase CFU-Mk increased from 31% ± 16% to 88% ± 14%). Moreover, the number of CFU-Mk per milliliter of marrow increased from 299 ± 269 to 842 ± 599. Despite this proliferative stimulus induced by GM-CSF on CFU-Mk, the number of circulating platelets was unchanged, suggesting that either megakaryocyte development or platelet release was not affected by the treatment.

To analyze the effect of GM-CSF treatment on morphologically recognizable megakaryocytes, two approaches were used.

First, a morphologic analysis was performed to investigate whether GM-CSF treatment could modify the maturation profile of megakaryocytes (Table 3). A significant decline in the percentage of mature (stage IV) megakaryocytes with a relative accumulation of the immature forms occurred during treatment.

Second, a search for specific GM-CSF receptors on megakaryocytes was made by incubating normal marrow cells with \(^{125}\)I-GM-CSF and subsequent analysis of labeled
cells. Megakaryocytes showed a significant labeling (Fig 1) that was partially decreased by incubating marrow cells with an excess of unlabeled GM-CSF (Table 4). This displacement of labeled GM-CSF, similar to that observed by Fraser et al., who studied the presence of erythropoietin receptors on marrow megakaryocytes, strongly suggests the presence of specific GM-CSF receptors on megakaryocytes.

DISCUSSION

These findings in subjects with normal hematopoiesis throw some light on the effect of GM-CSF in thrombocytopoiesis in vivo. Three observations suggest that GM-CSF plays a role in megakaryocytopoiesis:

1. There is a highly significant increase in the proliferative activity of CFU-Mk during subcutaneous GM-CSF administration. Based also on in vitro evidence, showing that GM-CSF alone supports the formation of megakaryocyte colonies, we suggest that GM-CSF stimulates CFU-Mk by directly acting on progenitors. However, the possibility of an indirect effect, caused by enhanced release of other cytokines (e.g., interleukin-6 [IL-6], etc) can not be ruled out.

2. GM-CSF treatment modifies the maturation profile of megakaryocytes, inducing a relative increase in the more immature forms.

Table 4. Binding of "OM-CSF (expressed as mean number of grains per cell ± SE) to Marrow Megakaryocytes From Normal Donors Not Undergoing GM-CSF Treatment

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total GM-CSF Bound</th>
<th>Nonspecific GM-CSF Binding</th>
<th>Specific Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>11 ± 0.9</td>
<td>4.9 ± 0.8</td>
<td>56%</td>
</tr>
<tr>
<td>Sample 2</td>
<td>19 ± 2.4</td>
<td>8.0 ± 1.4</td>
<td>53%</td>
</tr>
<tr>
<td>Sample 3</td>
<td>75 ± 5.1</td>
<td>44 ± 5</td>
<td>40%</td>
</tr>
</tbody>
</table>

Megakaryocytes were obtained from marrow buffy coat (samples 1 and 2) or after Percoll separation (sample 3). At least 50 megakaryocytes were counted for each point in samples 1 and 2 and more than 150 in sample 3.

(3) Incubation with labeled GM-CSF and autoradiography shows the presence of specific GM-CSF receptors on megakaryocytes.

However, despite these observations, treatment with GM-CSF did not alter the number of circulating platelets. This finding contrasts with the marked proliferative stimulus induced by GM-CSF through the granulocyte-monocyte line, resulting in a modulation in marrow composition with a rapid and marked granulo-monocytosis (Table 1).

Seeking an explanation for this difference in effect, it must be remembered that many cytokines promote (e.g., thrombopoietin, IL-6) or limit (e.g., transforming growth factor β) platelet production. In subjects with normal hematopoiesis, changes in marrow levels of these cytokines could offset the proliferative stimulation of megakaryocyte progenitors induced by pharmacologic doses of GM-CSF. This hypothesis may also explain why GM-CSF has an inconsistent effect on the restoration of platelet production when hematopoiesis has been affected (e.g., by chemotherapy, marrow transplantation). In these situations the final outcome of the proliferative stimulus of GM-CSF on early phases of megakaryocytopoiesis is less predictable because it depends on the number of residual progenitors and on endogenous cytokine levels.

In conclusion, our data show that GM-CSF action is restricted to early phases of megakaryocytopoiesis and does not influence platelet production in subjects with normal hematopoiesis. This result is presumably because of the fact that other stimulating or inhibiting factors are of decisive importance in the regulation of the intermediate and final stages of platelet production in vivo. In perspective, a sequential treatment with GM-CSF followed by cytokines acting at a late stage (presumably thrombopoietin or IL-6) might prove to stimulate platelet production in vivo.

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