The Mpl-Ligand or Thrombopoietin or Megakaryocyte Growth and Differentiative Factor Has Both Direct Proliferative and Differentiative Activities on Human Megakaryocyte Progenitors

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Previously, it was believed that megakaryocytopoiesis was regulated by two types of humoral factors: megakaryocyte colony-stimulating factor (MK-CSF), which acts on progenitors inducing their proliferation, and thrombopoietin (TPO), a megakaryocyte(s) (MK) maturation factor that induces platelet formation. The recently cloned Mpl-ligand (Mpl-L) seems to have both properties in vivo and in vitro and has also been called TPO. However, it cannot be excluded that a part of these activities is due to a synergistic effect with growth factors present in the serum or synthesized by accessory cells. To delineate the precise TPO (Mpl-L) biologic activities, we performed serum-free cultures at limiting cell dilution. Target cells were adult human marrow CD34+CD41+ cells, which represent a highly selected population of late MK progenitor or transitional cells. Cells were purified using a flow cytometer equipped with an automatic cloning design unit. We determined that the recombinant molecule had a biologic activity that reached a plateau at 10 ng/ml. At this concentration, a linear relationship between the average MK number per well and the number of cells seeded (between 1 to 50 cells per well) was observed. At one cell per well, 60% of the wells contained a single MK at day 5 of culture. Half of these wells contained only one large MK, whereas the other half contained several MK (up to 25), demonstrating that TPO has direct proliferative biologic activity. In contrast, at limiting dilution, none of the other cytokines tested (stem cell factor [SCF], interleukin-6 [IL-6], and erythropoietin [Epo]) were effective, whereas IL-3 showed a mild effect. However, a combination of SCF plus IL-6 plus IL-3 produced similar results as TPO alone. Addition of the other cytokines to TPO did not enhance the cloning efficiency of the CD34+CD41+ cells but increased twofold the average number of MKs per clone. MKs reached a ploidy of 32N and 64N in the presence of TPO. The mean ploidy value was approximately 6 and was not modified by addition of the other cytokines. At the ultrastructural level, a majority of the MKs showed maturational defects related to an imbalance between the synthesis of α-granules and demarcation membranes. However, a fraction (about 30%) had a cytoplasmic maturation that exactly mimicked that of marrow MKs. In addition, proplatelet-shedding MKs were observed in the cultures, even at limiting dilution. Such a result was not observed with any other individual cytokines, including the combination of three cytokines. This study shows that, at the unicellular level, TPO (Mpl-L) is both a proliferative and differentiative factor for MK progenitors.

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REGULATION OF megakaryocytopoiesis and platelet production is a complex phenomenon. It has been shown that numerous pleiotropic cytokines act in vivo and in vitro on megakaryocytopoiesis. In addition, for several years it has been suggested that platelet production is regulated by two elusive growth factors: megakaryocyte colony-stimulating factor (MK-CSF) and thrombopoietin (TPO), respectively. MK-CSF activity is present in sera from postirradiated animals and from patients with aplastic anemia. This factor primarily acts on MK progenitors by inducing MK colony formation in semisolid media or MK proliferation in liquid media. The blood level of this biologic activity correlates with the total MK mass but not with the platelet count. In contrast, TPO has been defined as a humoral factor acting essentially on late steps of megakaryocytopoiesis and unable to induce MK colony formation in vitro. The primary in vivo effect of this molecule is to increase MK volume and ploidy, leading to an enhancement in platelet production. In contrast with MK-CSF, the TPO level is inversely correlated with the platelet mass.

Using an antisense strategy, we showed that the product of the c-mpl gene, the Mpl receptor (Mpl-R), played a crucial role in human MK colony formation. In addition, these experiments suggested that the Mpl-ligand (Mpl-L) was present in sera from aplastic patients as well as in normal sera. Recently, Mpl-L was isolated by five independent groups using different approaches. The purified proteins were termed Mpl-L (Mpl-ligand), thrombopoietin, megakaryocyte growth and development factor (MGDF), or megapoietin. All purified proteins have the same sequence. Strong evidence that Mpl-L is the homeostatic regulator of thrombocytopoiesis is provided by Mpl-R knock-out mice, which present a severe but nonlethal thrombocytopenia.

In vitro experiments suggest that Mpl-L has the properties of MK-CSF and TPO for the following reasons: (1) the recombinant Mpl-L is capable of inducing MK colony formation and MK proliferation of both CD34+ human or purified murine hematopoietic stem cells; (2) the recombinant molecule induces polyploidization and a terminal MK maturation with proplatelet formation; and (3) the biologic level of the molecule is increased in plasma from animals made thrombocytopenic either by irradiation or by immune platelet destruction. In the latter case, the increase in the biologically active molecule follows the same kinetics as
DIRECT EFFECT OF MGDF ON MK PROGENITORS

data argue that Tpo and Mpl-L are the same molecule. Therefore, Mpl-L (TPO) appears to have a broad activity on MK differentiation. However, none of the published results have totally excluded that a part of the TPO activity is either indirect or related to a synergistic effect with other cytokines present in serum-containing cultures or synthesized by accessory cells. To delineate the biologic activity of TPO on human MK differentiation, we performed serum-free experiments at limiting cell dilution. This study focused on the CD34+CD41+ cell population, which represents a highly selected population of late MK progenitor cells, and investigated the effects of the recombinant molecule on their cloning efficiency, proliferation, polyploidization, and cytoplasmic maturation.

MATERIALS AND METHODS

Bone Marrow Cells

After receiving informed consent, bone marrow was obtained from normal adult donors during hip surgery. Samples were obtained in accordance with the institutional guidelines of the Committee on Human Investigation. Bone marrow cells were collected by vigorous shaking of bone fragments in Iscove’s modified Dulbecco’s medium (IMDM; Gibco, Paisley, UK) supplemented with 100 μg/mL of deoxyribonuclease DNase type I (Sigma, St Louis, MO). Cells were centrifuged once, counted, and separated on Ficoll-Hypaque (Seromed, Berlin, Germany). Light-density (less than 1.077 g/mL) cells (LDMC) were recovered and, after one washing, were used either for isolation of CD34+ cells by the immunomagnetic bead technique or by flow cytometry.

Antibodies

The anti-CD34 monoclonal antibody (MoAb) QBEND 10 (Immunotech, Lumigny, France) was used for immunomagnetic purification. Directly conjugated MoAbs R-PE-HPCA-2 (CD34) and fluorescein isothiocyanate (FITC)—anti-CD41a, purchased from Becton Dickinson (Mountain View, CA) and Immunotech, respectively, were used for flow cytometry. The Y2/51 MoAb (anti-CD61) was a gift from Dr D. Mason (Oxford, UK).

Isolation of CD34+ Cells by the Immunomagnetic Bead Technique

CD34+ cells were usually recovered from 2 × 108 LDMC. Cells were first incubated at 4°C for 30 minutes with the QBEND 10 MoAb at a concentration of 10 μg/mL and then with paramagnetic beads coupled to a goat antibody to mouse IgG (Dynabeads M-450; Dynal, Oslo, Norway) with a bead-to-target cell ratio of 5:1. CD34+ cells were isolated by magnetic separation and detached from the beads by chymopapain treatment (Sigma; 130 U/mL for 10 minutes). The CD34 epitope recognized by QBEND10 is cleaved by chymopapain. This treatment allows the collection of CD34+ cells free of beads and an immediate labeling with MoAbs that recognize chymopapain-resistant epitopes, such as R-PE HPCA2 and the FITC—anti-CD41a.

Cell Sorting

Cell sorting on the CD34 and CD41 differentiation markers was performed starting with immunomagnetic bead-isolated CD34+ cells. Cells were incubated simultaneously with the R-PE-HPCA2 (CD34) and FITC—anti-CD41a MoAbs. After one washing, cells were suspended in IMDM at a concentration of 5 × 10^6/mL and separated by cell sorting.

Cultures were sorted on a FACS Vantage flow cytometer (Becton Dickinson) equipped with an automatic cloning design. A morphologic gate including 80% of the events and all the CD34+ cells was determined on two-parameter histograms (side scatter [SSC] vs forward scatter [FSC]). Compensation for two-color labeled samples was set up with single-stained samples. Positivity or negativity for the CD41 antigen among the CD34+ cells was determined using control cells labeled with the PE-HPCA2 MoAb and an irrelevant IgG1 MoAb. Cells were sorted into CD34+CD41+ (average, 96% of the CD34+ cells) and CD34+CD41− cell fractions (average, 2% of the CD34+ cells).

For limiting dilution experiments, CD34+CD41− or CD41− cells were directly sorted into 96-well tissue culture plates (Falcon, Grenoble, France) using the automatic autoclone apparatus.

Human Cytokines

Cytokines were used alone or combined. Recombinant human (rh) interleukin (IL)-3 and rhIL-6 (gifts from Immunix, Seattle, WA) were both used at a final concentration of 100 U/mL (5 ng/mL and 5 ng/mL, respectively). Recombinant human erythropoietin (rh Epo), rh stem cell factor (rh SCF), and rh TPO (Mpl-L or MGDF) generously provided by Amgen (Thousand Oaks, CA), were usually used at a final concentration of 1 U/mL, 50 ng/mL, and 10 ng/mL, respectively.

Cell Cultures

CD34+CD41+ cells were cultured at a concentration of 2 × 10^4/mL or 1 × 10^5/mL in serum-free liquid medium in 96- (100 μL) or 24-well (500 μL) plates, as previously published. The medium contains insulin (100 ng/mL), 1.5% bovine serum albumin (BSA; Cohn’s fraction V; Sigma), sonicated lipids, and iron-saturated human transferrin and was supplemented with a different combination of cytokines. Cultures were studied from day 5 to day 7.

For single-cell cloning experiments, individual CD34+CD41+ or CD34−CD41+ cells were cultured at a concentration of 2 × 10^3 cells per 50 μL or 1 × 10^4 cells per 100 μL serum-free medium supplemented with different cytokines alone or in combination. Plates were examined on days 5 to 7 for the CD34+CD41+ cell subset and/or on days 5, 7, 10, and 13 for CD34−CD41− cells. Cultures were incubated at 37°C in a fully humidified atmosphere containing 5% CO2 in air, and each well was examined under an inverted microscope at a 40 × and 100 × magnification.

 Colony forming units-megakaryocyte (CFU-MK) were grown in semisolid medium using the same serum-free ingredients as in liquid culture and the fibrin clot technique. Cultures were studied after 7 days of growth. Colonies were quantified by an indirect immunoporphatase alkaline labeling technique using an anti-GPIIIa MoAb (CD61, Y2/51). Dishes were scanned under an inverted microscope.

MK Ploidy

MK ploidy was measured by a double-staining technique and flow cytometry. Cultured cells were counted, fixed with 2% paraformaldehyde (Serva, Heidelberg, Germany) for 20 minutes at 4°C, and then washed three times in phosphate-buffered saline solution without Ca2+ and Mg2+ (PBS; Gibco). Cells were kept at 4°C until analysis (less than 2 days). MKs were identified after labeling with an FITC-CD41a MoAb. DNA staining was performed by a 1-hour incubation in a propidium iodide solution (50 μg/mL, in isotonic sodium chloride containing 100 μg/mL RNase [Merck, Darmstadt, Germany] and 0.1% Tween 20 [Sigma]).

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Samples were analyzed on a FacsSort flow cytometer (Becton Dickinson). The cell flow rate was 500 to 1,000 cells per second. MKs were identified by the expression of CD41a. Controls were cells stained by an irrelevant FITC-IgG1 MoAb and propidium iodide. The frequency of MKs in different ploidy classes was usually evaluated on 5,000 MKs, or on the entire sample when MKs were less numerous (minimum, 2,500 MKs). The ploidy distribution was determined by setting markers at the nadirs between peaks.

**Ultrastructural Studies**

Cultured cells were studied by electron microscopy (EM). They were washed twice in Hanks’ medium at 4°C, fixed in 1.25% glutaraldehyde in Gey’s buffer for 10 minutes, washed, and incubated in diaminobenzidine medium. Cells were then postfixed with osmium tetroxide, dehydrated, and embedded in epon. Thin sections were examined with a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands) after lead citrate staining.

**RESULTS**

**Dose Response of CD34+ Cells to TPO**

Immunomagnetic bead-separated CD34+ cells (about 90% of purity) were cultured at a concentration of 2 × 10⁶/mL in serum-free medium (volume, 500 μL) to test the effects of TPO at concentrations ranging from 0.1 ng/mL to 50 ng/mL on the number of MKs and their mean ploidy level. After 10 days of culture, the absolute number of MKs as determined by flow cytometry had increased, and a plateau was reached at 5 ng/mL of the recombinant molecule (Fig 1A). Between 5 ng/mL to 50 ng/mL of TPO, the mean ploidy of mature MKs (those expressing the highest amounts of CD41a) as well as the number of MKs did not significantly change (Fig 1B). Therefore, we used a TPO concentration of 10 ng/mL in subsequent experiments.

**Maturational Activity of TPO on the CD34+CD41+ Cells**

**Light microscopy studies.** In the absence of growth factors, cells were lysed within 2 to 3 days. In the presence of TPO alone, large MKs intermingled with much smaller cells began to be detected after 2 days of culture (Fig 2A). The number of large MKs peaked between days 5 and 7, at which time proplatelet formation was observed (Fig 2B). At low cell concentration (2 × 10⁶/mL), 1% to 2% of the MKs simultaneously displayed long filamentous arms. However, because this process was quite short (about 30 minutes when a cell was examined under an inverted microscope), it was not possible to precisely enumerate the real number of platelet-shedding MKs. At higher cell concentration (1 × 10⁷/mL), proplatelet formation was much more frequently observed (5% to 10% of the MKs). MK lysis occurred during the following days.

When TPO was replaced by Epo, SCF, or IL-6, nearly all cells were lysed on day 7 of culture. Only rare MKs were observed. Intermediate results were observed in the presence of IL-3. In contrast, a combination of IL-3 plus IL-6 plus SCF gave very similar results to TPO alone. Proplatelet formation was also observed, but the phenomenon was much rarer and only observed at high cell concentration.

On cytopsin preparations, all cells grown with TPO had morphologic features of MKs. Although most MKs had an apparently normal maturation, some had obvious maturation defects, which included nuclear fragmentation and cytoplasmic membrane rupture. This observation explains the lysis of a fraction of MK without proplatelet formation.

**Ultrastructural studies.** Cells (2 × 10⁶/mL) were grown in the presence of TPO and were examined by EM at day 5 of culture. All the identifiable cells obtained from the CD34+CD41+ cell fraction belonged to the MK lineage, with a majority of mature cells intermingled with immature MKs or blasts without MK organelles. Overall, the quality of the maturation for cultured MKs was good. However, among the mature MKs, two thirds displayed some maturation defects, which were characterized either by an excess of a-granules with rare demarcation membranes (DM; Fig 3A) or inversely by an excess of flat demarcation membranes with dispersed α-granules (Fig 3B). The remaining one third had a normal maturation similar to that of marrow MKs. DM and α-granules were present and normally distributed in the cytoplasm (Fig 3C). Multivesicular granules, which are usually ex-
tremely abundant in cultured MKs, were rare. Addition of other cytokines (IL-3, IL-6, and SCF, alone or in combination) did not further improve MK maturation.

In contrast, a normal MK maturation was never observed at low cell concentration (2 × 10^7/mL) when TPO was omitted from the culture medium and even when a combination of three cytokines (IL-3, IL-6, and SCF) was used.

**Ploidy.** MK ploidy was studied by flow cytometry in double-staining experiments. In the presence of TPO, nearly all cells (greater than 90%) were labeled by the antibody against glycoprotein (GP) IIb/IIIa (CD41a), and a large majority (greater than 80%) expressed very high amounts of these glycoproteins, which correlated with ploidy. Apoptotic cells detected as events with a ploidy below 2N were rare (less than 10%) at day 5. A typical ploidy histogram is shown in Fig 4A. The majority of MKs belonged to the 2N, 4N, and 8N ploidy classes, but MKs with a 32N or 64N DNA level were always observed. In addition, in one of the four repeated experiments, some 128N MKs (1%) were also detected. Addition of Epo, IL-6, IL-3, or SCF (Fig 4B) to TPO did not significantly change the ploidy histogram or the mean ploidy.

In the presence of Epo, SCF, or IL-6 alone, numerous apoptotic cells (up to 80%) were present. The rare MKs grown in the presence of these cytokines had lower CD41a amounts than those grown with TPO, and analysis of their ploidy showed a distribution in all ploidy classes below 16N. In the presence of IL-3, more MKs were present when compared with the previous cytokines. The ploidy histogram showed a lower frequency of 2N MKs than with TPO, but MKs were not observed with a ploidy over 16N. In contrast, when three cytokines (IL-6, IL-3, and SCF) were present in the culture, the ploidy histogram was similar to that observed with TPO alone. However, 64N and 128N classes were never detected.

**Proliferative Activity of TPO on the CD34⁺CD41⁺ Cells**

**Semisolid assays.** In a first set of experiments, CD34⁺CD41⁺ cells were analyzed for their abilities to form MK colonies in semisolid medium containing TPO. Individual MKs as well as clusters of two to three MKs and colonies comprising several MKs were detected (Table 1). TPO had a higher MK colony-stimulating activity than IL-3. The numbers of MK colonies, clusters, or individual MKs were not significantly modified by the addition of SCF, IL-3, or the combination of the three cytokines (IL-3 + SCF + IL-6). However, the size of the colonies (MK number per colony) was slightly increased by the addition of three cytokines: colonies of more than 10 cells were only detected in this culture condition. To perform a more complete analysis, limiting dilution experiments were performed.

**Limiting dilution experiments.** First, CD34⁺CD41⁺ cells were plated at a concentration of 1 to 50 cells in 100 μL volume in the presence of TPO alone or SCF plus TPO. The average number of cells per well was determined by observation of the cultures under an inverted microscope. As shown in Fig 5, a linear relationship was found between the average number of cells per well and the number of cells plated. In addition, the slopes of the two straight lines were similar. Then, four independent experiments were performed at one cell per well (a total of 1,200 wells). The percentage of positive wells, as well as the number of cells per well, was determined on day 5 in culture. Results are shown in Fig 6. In the absence of growth factor, all wells were devoid of cells. In contrast, in the presence of TPO alone, the percentage of positive wells was approximately 60%. Among these positive wells, 50% contained an individual large MK, whereas in the others, a proliferation had occurred because clones containing up to 25 identifiable MKs were observed. On the average, the clones that contained the greatest number of cells were composed of smaller cells. Some platelet-shedding MKs were observed in these limiting dilution experiments.

In subsequent experiments, we compared the effects of TPO to those of SCF, IL-6, Epo, or IL-3. In the presence of SCF, IL-6, or Epo, the percentage of positive wells was less than 10%, and no clones contained more than three cells. In contrast, in the presence of IL-3, the cloning efficiency was 20%, and some clones comprised up to eight cells. Therefore, we compared the effects of TPO alone with those of a combi-
Fig 3. Ultrastructural studies of MKs grown from CD34^+CD41^+ cell suspension after 5 days in serum-free conditions with TPO alone. In panels A and B, large MKs with an abnormal maturation are shown, whereas in panel C, an MK with a normal maturation is shown. (A) Abundant α-granules (αGr) are clustered in the center; at the periphery, a few rudimentary demarcation membranes (DM) are seen. Original magnification (OM), × 10,300. (B) Large development of DM is shown, with some being delated (arrows) while the others have no internal channels (arrowheads). The α-granules (αGr) are pushed outside the membrane zones. OM, × 15,000.
nation of cytokines. The percentage of positive wells and the average cell number per clone were similar in the presence of TPO, of SCF plus TPO, and of IL-3 plus IL-6 plus SCF. In contrast, the combination of IL-3, IL-6, SCF, and TPO did not change the cloning efficiency but increased twofold the cell number per clone.

**Proliferative Activity of TPO on the CD34⁺CD41⁻ Cells**

The effects of TPO were subsequently tested in the CD34⁺CD41⁻ cell population, which represents a population of more immature MK progenitors and contains all the different lineage progenitors. When cells were grown at a concentration of $1 \times 10^4$/mL in the presence of TPO alone, MKS were also obtained in suspension. The MK wave of differentiation was detected slightly later (beginning at day 5) than with cultured CD34⁺CD41⁺ cells, and MKs could be analyzed until day 13 of culture. A large fraction of the cells (up to 70%) obtained in these cultures expressed CD41a. In contrast to the CD34⁺CD41⁺ cell subset, there was a marked synergistic effect of TPO with SCF or IL-3 (Fig 7). A similar result was observed when the unFractionated CD34⁺ cell population was studied (data not shown).

Finally, in four independent experiments performed at limiting dilution, effects of TPO were tested on the CD34⁺CD41⁻ cell subset. Surprisingly, the percentage of positive wells was high (24% ± 3%) for such a heterogenous population. As for the CD34⁺CD41⁻ cells, half of the positive wells contained only one large MK. The distribution of the cell numbers in the other clones slightly differed from the CD34⁺CD41⁻ cell population as rare wells contained up to 50 cells. Notably, optimal growth occurred slightly later (days 7 to 10) than with the CD34⁺CD41⁻ population, and small cells that could not be identified were often observed.

**DISCUSSION**

The aim of this study was to determine the effects of TPO (Mpl-L or MGDF) on human MK progenitors. A large part of this study has been performed on a CD34⁺CD41⁻ cell population. These cells represent a rare subset of the marrow CD34⁺ cells (1% to 5%). One of the pitfalls in studying this cell subset could be a possible false labeling due to the binding of platelets or platelet fragments to CD34⁺ cells by specific receptors during the purification procedure. This is especially true when working with CD34⁺ cells isolated.
from blood leukapheresis or total blood samples. For these reasons, we have previously reported the biologic properties of marrow CD34⁺CD41⁻ cells that were selectively amplified during 1 week in in vitro liquid cultures. We have shown that the immunomagnetic bead separation technique followed by a chymopapain treatment to detach cells from the beads also released most platelet fragments from the CD34 cells and allowed a direct study of the CD34⁺CD41⁻ cell population. These cells are a highly selected population of late MK progenitors that differentiate in less than 7 days and may be the main target cells for TPO because, by fluorescence labeling, a large number of them express the Mpl-R. Using this cell population, the reported data demonstrate that TPO is both an MK-CSF and a maturational factor.

On individual cells, TPO was able to induce a proliferation; clones comprising up to 25 MKs were observed. Because serum-free cultures were used, this result strongly suggests that TPO is a proliferative factor by itself and does not require any other cytokines. The only limitation concerns the contamination of our serum-free medium by BSA, which may contain undefined growth factors. In addition, insulin was included in the components of our medium, but its removal did not abolish the proliferative effects of TPO (data not shown).

Notably, most of the clones obtained from individual CD34⁺CD41⁻ cells only contained one large MK. This is not related to a particular biologic property of TPO, because similar results were observed with a combination of cytokines, nor is it related to the target cells. Indeed, similar results were obtained with the CD34⁺CD41⁻ cell population, which includes more primitive MK progenitors. Therefore, this result may suggest that the commitment to polyploidy of MK progenitors is independent of their generation age or of their stage of differentiation as previously reported.

TPO is also a differentiative factor. MKs obtained in vitro from CD34⁺CD41⁻ cells mature to platelet-shedding. High-ploidy MKs (up to 64N and occasionally 128N) were present in the cultures. Although some MKs had an abnormal cytoplasmic maturation, a fraction (about 33%) of the cultured MKs had an overall morphology indistinguishable from marrow MKs as demonstrated by EM examination. Such a differentiative effect was also observed at the unicellular level, as large MKs, as well as occasional platelet-shedding MKs, were seen. However, induction of proplatelet formation in our culture conditions was much less effective than that recently reported in culture from peripheral blood CD34⁺ cells by a two-step culture procedure. However, this phenomenon was greatly improved by increasing the cell concentration, suggesting that some other factor(s) may be required to induce platelet shedding.

Using a double-staining technique to measure MK ploidy, we found that a significant fraction of 2N and 4N MKs were present in cultures supplemented with TPO alone. These low-ploidy MKs did not always correspond to immature cells. Indeed, whatever the day of culture chosen for ploidy measurement (day 5 or day 7), the 2N and 4N classes were

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Table 1. Effects of Growth Factors on the Plating Efficiency of CD34⁺CD41⁻ Cells

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>1 Cell</th>
<th>2-3 Cells</th>
<th>4-5 Cells</th>
<th>6-9 Cells</th>
<th>≥10 Cells</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.73 ± 0.6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>TPO</td>
<td>247.7 ± 22.4</td>
<td>93.3 ± 13.5</td>
<td>12.2 ± 5</td>
<td>1.4 ± 1.6</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>65.1 ± 26.3</td>
<td>6.6 ± 4.4</td>
<td>0.36 ± 0.63</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TPO + IL-3</td>
<td>112.5 ± 24.1</td>
<td>67.3 ± 10</td>
<td>14 ± 8.3</td>
<td>2.5 ± 1.6</td>
<td>0</td>
</tr>
<tr>
<td>SCF</td>
<td>21.7 ± 16.7</td>
<td>1.1 ± 1.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TPO + SCF</td>
<td>281.1 ± 63</td>
<td>80.7 ± 4.4</td>
<td>15.1 ± 6.7</td>
<td>5.6 ± 1.1</td>
<td>0</td>
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<tr>
<td>IL-3 + SCF + IL-6</td>
<td>121.4 ± 11.8</td>
<td>44.7 ± 19.8</td>
<td>3.6 ± 0.63</td>
<td>0.3 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>TPO + IL-3 + SCF + IL-6</td>
<td>204.4 ± 14.2</td>
<td>92.8 ± 20.8</td>
<td>13.3 ± 2.8</td>
<td>2.8 ± 2.2</td>
<td>1.66 ± 0.57</td>
</tr>
</tbody>
</table>

CD34⁺CD41⁻ cells (500/mL) were grown by the serum-free fibrin clot technique (SCF, 50 ng/mL; IL-3, 100 U/mL; IL-6, 100 U/mL; TPO, 20 ng/mL). Cultures were stopped at day 7 and labeled by an anti-CD61 MoAb and the immunoperoxidase technique. The results are the mean number of cells ± SD from a triplicate culture and are expressed per 1,000 CD34⁺CD41⁻ cells.
Fig 5. Relationship between the number of cells per well obtained at day 5 and the number of cells plated at limiting dilution. CD34+CD41+ cells were sorted into the 96 wells of a tissue culture plate using the automatic cloning design of a flow cytometer at 1, 5, 10, 20, and 50 cells per well. The number of cells per well after 5 days of culture was evaluated under an inverted microscope. The curve represents the results of one experiment performed in triplicate.

Therefore, TPO exerts a broad activity (proliferation and differentiation) on megakaryocytopoiesis that differs from all other cytokines tested in this study (IL-3, IL-6, SCF, Epo). SCF, IL-6, or Epo showed a very low effect on MK survival and differentiation at the unicellular level. IL-3 had some activity but was much less effective than TPO. In contrast, a combination of three cytokines (IL-3, IL-6, and SCF) could partly replace the TPO activity. The major differences were the inability of this cytokine combination to promote the highest ploidy classes and to induce a complete cytoplasmic maturation at low cell concentration. As a consequence, proplatelet formation appeared to occur more rarely than with TPO. However, as mentioned above, our in vitro assay is not a quantitative measure of proplatelet formation, and further experiments are required to understand whether these differences are significant.

The biologic activity of TPO on megakaryocytopoiesis can be easily compared to that of Epo on erythropoiesis. At the level of CD34+CD41+ cells, a cell type that has many similarities with CFU-E in the erythropoietic pathway, TPO has a direct effect. Addition of other cytokines moderately increases or does not enhance TPO activity when this cytokine is used at an optimal concentration. Similarly, the effects of Epo at full dose are not synergized by other cytokines on CFU-E. In contrast, despite a direct action on more immature MK progenitor cells (CD34+CD41+), the effects of TPO are markedly synergized by IL-3 and SCF, as described for Epo on burst-forming units-erythroid (BFU-E). A synergistic effect with SCF, Epo, or IL-11 with TPO, as well as an additive effect between IL-3 and TPO, has been recently observed for murine CFU-MK. Therefore, TPO and Epo have not only some homology in their structure, but also in their biologic activities.

Previous experiments have clearly shown the presence of...
a feedback loop between MK or platelets and the plasma level of TPO, suggesting that TPO is a physiologic regulator of platelet production.\(^{18,35}\) This assumption was reinforced by the presence of a severe thrombocytopenia in Mpl-R knock-out mice.\(^{15}\) The present results demonstrate that TPO acts directly on the different steps of megakaryocytopoiesis. This strongly suggests that TPO is probably the unique mediator of homeostatic platelet production, because this process does not require the presence of other cytokines. However, it remains to determine whether TPO is a totally MK lineage-specific factor.

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**Fig 7.** Effects of TPO, IL-3, and SCF on the MK differentiation of CD34+CD41+ cells. Suspension cultures of CD34+CD41+ cells were performed in 500 µL of serum-free culture medium but at a low cell concentration (1 x 10^4/mL). Cultures were twice diluted at day 7 and were studied by flow cytometry at day 10. The percentage of MKs was determined as the number of cells expressing CD41a. Their absolute number was calculated by multiplying the number of cells by the proportion of CD41a+ cells. The absolute number of MKs for each individual cytokine or combination of cytokines was compared with that obtained with TPO alone, and the ratio was calculated in percentage. Results represent the average ± SEM of three independent experiments.
DIRECT EFFECT OF MGDF ON MK PROGENITORS


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The Mpl-ligand or thrombopoietin or megakaryocyte growth and differentiative factor has both direct proliferative and differentiative activities on human megakaryocyte progenitors

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