Thrombopoietin, But Not Erythropoietin, Directly Stimulates Multilineage Growth of Primitive Murine Bone Marrow Progenitor Cells in Synergy With Early Acting Cytokines: Distinct Interactions With the Ligands for c-kit and FLT3

By Veslemøy Ramsfjell, Ole J. Borge, Ole P. Veiby, José Cardier, Martin J. Murphy Jr, Stewart D. Lyman, Si Lok, and Sten E.W. Jacobsen

The hematopoietic system is organized in a hierarchical fashion, with the infrequent pluripotent hematopoietic stem cell at the top capable of replenishing all cell lineages in the blood. The growth, differentiation, and viability of hematopoietic progenitors and stem cells are regulated in a complex manner involving cell-cell interactions as well as soluble hematopoietic growth factors (HGFS). An important, if not essential, phenomenon in early hematopoiesis is synergy among HGFS, in that most primitive progenitors can only be recruited to proliferate in vitro by the combined stimulation of multiple HGFS. In contrast, progenitors committed to different cell lineages can often be recruited by stimulation with a single cytokine, although synergy between multiple cytokines might result in enhanced proliferation of committed progenitor cells as well.

The complex development of megakaryocytes (Mks) from their bone marrow (BM) precursors is one of the least understood aspects of hematopoiesis. The recent cloning of thrombopoietin (Tpo), the ligand for c-mpl, has been shown to be the principal regulator of megakaryocytopoiesis and platelet production. The ability of Tpo to potentially stimulate the growth of committed megakaryocyte (Mk) progenitor cells has been studied in detail. Murine fetal liver cells, highly enriched in primitive progenitors, have been shown to express c-mpl, but little is known about the ability of Tpo to stimulate the growth and differentiation of primitive multipotent bone marrow (BM) progenitor cells. Here, we show that Tpo alone and in combination with early acting cytokines can stimulate the growth and multilineage differentiation of Lin- Sca-1+ BM progenitor cells. In particular, Tpo potently synergized with the ligands for c-kit (stem cell factor [SCF]) and flt3 (FL) to stimulate the increase in the number and size of clones formed from Lin- Sca-1+ progenitors. When cells were plated at 1 cell per well, the synergistic effect of Tpo was observed both in fetal calf serum-supplemented and serum-depleted medium and was decreased if the addition of Tpo to cultures was delayed for as little as 24 hours, suggesting that Tpo is acting directly on the primitive progenitors. Tpo added to SCF + erythropoietin (Epo)-supplemented methylcellulose cultures potently enhanced the formation of multilineage colonies containing granulocytes, macrophages, erythrocytes, and Mks. SCF potently enhanced Tpo-stimulated production of high-ploidy Mks from Lin- Sca-1+ progenitors, whereas the increased growth response obtained when combining Tpo with FL did not translate into increased Mk production. The ability of Tpo and SCF to synergistically enhance the growth of Lin- Sca-1+ progenitors was predominantly observed in the more primitive rhodamine 123" fraction. Tpo also enhanced growth of Lin- Sca-1+ progenitors when combined with interleukin-3 (IL-3) and IL-11 but not with IL-12, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, or Epo. Epo, which has high homology to Tpo, was unable to stimulate the growth of Lin- Sca-1+ progenitors alone or in combination with SCF or FL, suggesting that c-mpl is expressed on more primitive stages of progenitors than the Epo receptor. Thus, the present studies show the potent ability of Tpo to enhance the growth of primitive multipotent murine BM progenitors in combination with multiple early acting cytokines and documents its unique ability to synergize with SCF to enhance Mk production from such progenitors.

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1+ progenitor cells in combination with other early acting cytokines, resulting in recruitment of additional progenitors as well as enhanced proliferation of individual progenitors. This growth-promoting activity of Tpo results in multipotent progenitors producing high numbers of polyplid Ms as well as other (myeloid/erythroid) cell lineages.

MATERIALS AND METHODS

HGFs. Recombinant human flt3 (rHuFL) and recombinant murine Tpo (rMuTpo) were generated as previously described. Recombinant rat SCF (rSCF), rHu granulocyte colony-stimulating factor (rHuG-CSF), and rMu granulocyte-macrophage CSF (rMuGM-CSF) were kindly provided by Dr I. McNiece (Amgen Corp, Thousand Oaks, CA) and purified rMuIL-3 was from Peprotech Inc (Rocky Hill, NJ). rHuIL-11 and rMuIL-12 were gifts from Genetics Institute (Cambridge, MA). rHuEpo was a generous gift factor (rHuG-CSF), and rMu granulocyte-macrophage CSF (rMuGM-CSF) were kindly provided by Dr T. Kina, Kyoto, Japan). Cells were washed once. Sheep antirat IgG (Fc)-conjugated immunomagnetic beads (Dynal, Oslo, Norway) were added to a cell/bead ratio of 1:2 and incubated at 4°C for 45 minutes on a mixing wheel. The magnetic beads were removed with a magnetic particle concentrator (MPC-6; Dynal), and the unattached cells were transferred to a second tube containing the same (absolute) amount of magnetic beads and incubated for another 30 minutes as before.

Enrichment and purification of Lin 'Sca-1' BM cells. Lin cells were isolated from normal C57Bl/6 mice according to a previously described protocol. Briefly, femurs and tibiae were gently crushed in a mortar. The cell suspension was filtered through a 70-μm mesh filter, and cells were counted in a hematocytometer and concentrated in a volume of either complete IMDM or serum-depleted medium (x-vivo 15; BioWhittaker) supplemented with 1% (v/v) fetal calf serum supplemented with 20% fetal calf serum (FCS; BioWhittaker), penicillin, and L-glutamine (complete IMDM) were incubated without Rh123 for 20 minutes at 37°C and the Lin-Sca-l+ with lowest (25%; Rh123 uptake) and highest Rh123 uptake (25%; Rh123) were isolated as previously described. The same protocol as described above for the enrichment of Lin 'Sca-1' cells was followed except that Lin' cells were incubated with biotinylated rat anti-mouse Ly-6A/E antibody (Pharmingen) or an isotype-matched control antibody. The cells were washed, and Lin 'Sca-1' cells were sorted on a Coulter Epics Elite Cell Sorter (Coulter Electronics, Hialeah, FL). The final recovery of Lin 'Sca-1' cells from unfractionated BM cells was approximately 0.05%. Reanalysis of sorted Lin 'Sca-1' cells reproducibly showed a purity greater than 95%.

AchE is a marker enzyme of murine Ms. Briefly, cultured cells were stained directly in almost completely dried wells. Cells were incubated with acetylcholine substrate overnight at 37°C and 5% CO2 in air and scored on an inverted microscope. Ms were isolated as colonies containing three or more Ms. Cells were stained as follows: E, erythroid colonies; GM, granulocyte/macrophage colonies; Mk, megakaryocyte colonies; GEM, granulocyte/erythrocyt/macrophere colonies; GMMk, granulocyte/macrophage/megakaryocyte colonies; GEMMs, granulocyte/erythrocyt/macrophere/megakaryocyte colonies; and Blast, blast cell colonies.

Acetylcholinesterase (AchE) staining. AchE activity was detected as previously described. Briefly, Lin 'Sca-1' cells were cultured in serum-depleted medium with different cytokine combinations. At day 11, the cells were stained directly in almost completely dried cells. Cells were incubated with acetylcholine substrate overnight at 37°C and 5% CO2 in air and scored on the basis of color change. The Ms phenotype was also confirmed by parallel experiments in which individual colonies were transferred to slides in a cytospin centrifuge, fixed, and Giemsa-stained.

Cell morphology. Multiple Lin 'Sca-1' cells were plated in serum-depleted medium supplemented with predetermined optimal concentrations of cytokines at 37°C and 5% CO2 in air, at which time the total cell numbers were determined. Cell morphology was examined after Giemsa staining of cytospin preparations.

Measurement of Ms ploidy. Lin 'Sca-1' cells were cultured in serum-depleted medium supplemented with cytokines at predetermined optimal concentrations. After 9 to 11 days of incubation at 37°C and 5% CO2 in air, cells were analyzed by 2-color flow cytometric analysis using the murine Ms-specific rat monoclonal antibody 4A5 and propidium iodide as a measure of cellular DNA content.

Briefly, cultured cells were stained with 4A5 or an isotype-matched control rat IgG2 antibody (Pharmingen) for 30 minutes on ice and then washed twice in Dulbecco’s phosphate-buffered saline with 1% FCS. The cells were next incubated for 5 minutes with ChromPure goat IgG whole molecule (Jackson ImmunoResearch Labs, Inc, West Grove, PA) to block unspecific binding of the secondary antibody. Next, the cells were incubated for 30 minutes on ice with fluorescein isothiocyanate-conjugated goat F(ab')2 antirat IgG (Southern Biotechnology Associates, Inc, Birmingham, AL). Cells were washed twice and resuspended in a solution consisting of 0.1% sodium citrate, 0.1% Triton X-100, 50 U/mL RNase A, and 10 μg/mL propidium iodide (all from Sigma, St Louis, MO). 4A5+ cells (Ms) were identified by flow cytometry (FACSort; Becton Dickin-
Table 1. The Effect of Tpo on Clonal Growth of Lin"Sca-1" Progenitor Cells

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Clone Size*</th>
<th>Total Clones/300 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Medium</td>
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<tr>
<td>Tpo</td>
<td>2 (1)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>FL</td>
<td>18 (1)</td>
<td>5 (0)</td>
</tr>
<tr>
<td>Tpo+FL</td>
<td>22 (2)</td>
<td>27 (3)</td>
</tr>
<tr>
<td>SCF</td>
<td>6 (1)</td>
<td>9 (1)</td>
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<tr>
<td>Tpo+SCF</td>
<td>17 (1)</td>
<td>25 (6)</td>
</tr>
<tr>
<td>FL+SCF</td>
<td>25 (2)</td>
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<td>15 (4)</td>
<td>60 (3)</td>
</tr>
<tr>
<td>IL-11</td>
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</tr>
<tr>
<td>Tpo+IL-11</td>
<td>11 (2)</td>
<td>7 (3)</td>
</tr>
<tr>
<td>FL+IL-11</td>
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<td>39 (2)</td>
</tr>
<tr>
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<td>8 (0)</td>
<td>56 (2)</td>
</tr>
<tr>
<td>IL-12</td>
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<tr>
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<td>27 (4)</td>
</tr>
<tr>
<td>Tpo+FL+IL-12</td>
<td>23 (3)</td>
<td>30 (2)</td>
</tr>
</tbody>
</table>

Lin"Sca-1" BM cells were isolated as described in Materials and Methods, plated at 1 cell/well in 20 μL complete IMDM, and supplemented with predetermined optimal concentrations of cytokines (see Materials and Methods). Wells were scored for degree of cell growth after 10 to 12 days of incubation at 37°C and 5% CO₂ in humidified air. The results represent means (±SEM) of three separate experiments. The statistical significance of differences between group means were determined using the Student’s t-test.

* Scoring criteria: 1. 3 to 50 cells; 2. >50 cells to 10% of the well covered by cells; 3. cells covering >10% to 50% of the well; 4. >50% of the well covered by cells.

† P < .05.

Son, San Jose, CA. The percentage of 4A5⁺ cells identified in cultures were compared and found to be similar to the percentage of MKs scored morphologically after Giemsa staining of cytospin preparations. The 2N and 4N peak positions were identical to those of un gated cells.

Statistics. All results were expressed as the mean (±SEM) of data obtained from three or more separate experiments. The statistical significance of differences between group means were determined using the Student’s t-test.

RESULTS

Tpo synergistically and directly enhances the clonal growth of Lin"Sca-1" progenitor cells in combination with other early acting cytokines. To study the ability of Tpo to directly modulate the growth of primitive progenitors, Lin"Sca-1" cells were plated at a concentration of 1 cell/well in complete IMDM supplemented with Tpo (2,000 U/mL), alone or in combination with other cytokines (Table 1). Alone, Tpo induced little or no clonal growth of Lin"Sca-1" cells. However, Tpo synergized with FL to increase the cloning frequency of Lin"Sca-1" progenitor cells by 127% (from 22 to 50 clones). Similarly, Tpo enhanced SCF-stimulated clonal growth of Lin"Sca-1" cells by 200%. Furthermore, Tpo significantly increased the number of clones formed in response to the potent combination of FL+SCF by 52% (from 63 to 96 clones). The stimulatory effect of Tpo on FL-induced clonal growth of Lin"Sca-1" cells was concentration-dependent, with half maximum stimulation observed at 50 U/mL and maximum stimulation observed at 250 U/mL (Fig 1). The same concentration-dependent response was observed for Tpo-induced growth stimulation in response to FL+SCF (Fig 1). Interestingly, whereas IL-11 alone had little or no growth-stimulatory effect on Lin"Sca-1" cells, the cloning frequency was enhanced (from 1 to 18 clones) on the addition of Tpo (Table 1). In contrast, no growth was observed in response to the combination of Tpo and IL-12. In agreement with previous studies, FL potently synergized with IL-11 and IL-12, and Tpo further increased the clonal growth in response to these two cytokine combinations by 59% and 42%, respectively. Thus, Tpo directly and synergistically enhances the cloning frequency of Lin"Sca-1" progenitor cells when acting in combination with multiple early acting cytokines. Tpo in combination with other cytokines also increased the size of the clones formed by Lin"Sca-1" progenitor cells (Table 1). FL alone stimulated predominantly the formation of clusters (<50 cells), whereas Tpo potently increased the number of FL-induced colonies sixfold (from 5 to 28 colonies). Six clusters and 10 colonies were formed in response to SCF alone, which increased to 17 clusters and 30 colonies on the addition of Tpo. Whereas no colonies and only one cluster were observed in response to IL-11, 11 clusters and 7 colonies were formed on the addition of Tpo. When Tpo was combined with potent 2-factor combinations (FL+SCF, FL+IL-11, or FL+IL-12), there was a preferential increase in the number of clones observed at 50 U/mL and maximum stimulation observed at 250 U/mL (Fig 1).

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![Fig 1. Concentration-dependent response of Tpo-stimulated colony formation of Lin"Sca-1" progenitor cells. Lin"Sca-1" cells were plated at a density of 1 cell/well in 20 μL complete IMDM supplemented with FL (50 ng/mL), alone or in combination with SCF (50 ng/mL), and were exposed to increasing concentrations of Tpo as indicated. Wells were scored for clonal growth after 10 to 12 days of incubation at 37°C and 5% CO₂ in air. Results represent means (±SEM) of three separate experiments.](https://www.bloodjournal.org/content/4483.full)
of larger colonies (>10% of the well covered). Thus, the ability of Tpo to stimulate the growth of Lin Sca-1 progenitor cells is due to recruitment of additional progenitors as well as enhanced proliferation within individual clones.

Effects of Tpo on the growth of Lin Sca-1 progenitors in serum-depleted medium. To examine whether the ability of Tpo to stimulate the growth of Lin Sca-1 progenitors was dependent on the presence of factors present in FCS, we next examined the effects of Tpo on Lin Sca-1 clonal growth in a serum-depleted (containing 1% detoxified BSA) medium (Fig 2). Whereas Tpo alone induced little or no clonal growth in FCS-containing medium, 12 clusters and 1 colony were observed in response to Tpo alone in serum-depleted medium. Similarly, the number of SCF-stimulated clones increased by 91% in serum-depleted cultures (while, in fact, their sizes were reduced), whereas the clonal formation induced by FL, IL-11, or IL-12, was the same in both media. Similar to that in the FCS-containing cultures (Table 1), Tpo enhanced clonal growth in combination with FL and SCF (threefold and twofold stimulation, respectively). The number of clones formed in response to FL+SCF, FL+IL-11, and FL+IL-12 were also enhanced by Tpo, resulting in a cloning frequency of 33% to 37%. Furthermore, as in FCS-supplemented cultures, the average size of the clones formed in response to these cytokine combinations was increased on the addition of Tpo, and, accordingly, the most dramatic increase was observed in large colonies (Fig 2). Therefore, Tpo stimulates the growth of Lin Sca-1 progenitor cells both in serum-supplemented and serum-depleted cultures.

Interaction between Tpo and CSFs on the growth of Lin Sca-1 progenitor cells. Tpo has been shown to enhance the clonal growth of committed BM progenitor cells in combination with Epo or IL-3.16 To investigate whether Tpo could also affect the growth of primitive progenitor cells in combination with Epo, IL-3, or other CSFs, Lin Sca-1 cells were cultured at a concentration of 1 cell/well in serum-depleted medium with CSFs or Epo, in the absence or presence of Tpo. Tpo alone stimulated growth of 11 of 300 Lin Sca-1 cells (Fig 3). IL-3 induced the formation of 27 clones, whereas 45 clones were formed when Tpo was added. In particular, an increase was observed in the number of colonies (>50 cells; from 16 to 27). In contrast, when Tpo was combined with G-CSF, Epo, or GM-CSF, clonal growth was not significantly enhanced with regard to number or size.

Because of the high homology between Tpo and Epo, and their corresponding receptors, as well as the synergistic interaction reported between the two cytokines on unfractionated BM cells,9,16,32-34 we compared in more detail the
TPO PROMOTES GROWTH OF PUTATIVE STEM CELLS

Fig 4. Comparison between the ability of Epo and Tpo to enhance clonal growth of Lin‘Sca-1’ progenitor cells. Lin‘Sca-1’ cells were plated at 1 cell/well in 20 μL serum-depleted medium, with indicated cytokines at predetermined optimal concentrations as described in Materials and Methods. Clonal growth (total colonies and colonies >50 cells) was scored after 10 to 12 days incubation at 37°C and 5% CO₂ in air. Results are presented as means (±SEM) of four separate experiments. *, No clones observed. Student’s paired t-test was performed comparing colony formation in the absence or the presence of either Tpo or Epo. *, P < 0.05; **, P < .005.

ability of Tpo and Epo to enhance the growth of Lin‘Sca-1’ progenitor cells (Fig 4). No clonal growth was observed in response to Epo alone, whereas Tpo stimulated the growth of 12 of 300 Lin‘Sca-1’ cells. Furthermore, in contrast to the significant enhancement of FL- and SCF-induced clonal growth (both with regard to number and size) observed in response to Tpo, Epo did not enhance the clonal formation of Lin‘Sca-1’ progenitors in combination with either SCF or FL. Thus, unlike Tpo, Epo appears to have no ability to enhance the clonal growth of Lin‘Sca-1’ progenitors.

The ability of Tpo to synergize with FL on Lin‘Sca-1’ progenitors requires the early action of Tpo. The synergy between FL and Tpo could potentially be caused by a sequential action of the two cytokines in that the early acting FL might simply initiate proliferation and, subsequently, Tpo might exert its synergistic activity at a later stage of differentiation. Thus, to investigate whether the synergistic activity required the presence of Tpo from the initiation of culture, a kinetic study was performed in which a 24- or 48-hour preincubation of Lin‘Sca-1’ cells with FL followed by the addition of Tpo was compared with the growth observed when both Tpo and FL were added at initiation of culture (Fig 5). A 24-hour delayed addition of Tpo to FL-stimulated cultures reduced the total number of clones by 41%, as compared with the cultures to which the two cytokines were added simultaneously. If the addition of Tpo to FL-preincubated cultures was delayed for 48 hours, the clonogenic frequency was reduced to that of cells stimulated by FL only.

However, even if delayed for 48 hours, the addition of Tpo still significantly (P < .05) increased the size of the remaining clones (Fig 5). Thus, the ability of Tpo to optimally recruit Lin‘Sca-1’ progenitor cells to proliferate requires its presence from the initiation of culture.

The effect of Tpo on MK formation and multilineage differentiation of Lin‘Sca-1’ progenitor cells. Lin‘Sca-1’ cells plated at a concentration of 1 cell/well were next analyzed for their ability to form MKs as well as other mature cells in serum-depleted medium in the presence and absence of Tpo in combination with other cytokines (Table 2). A total of 50% of the clones formed in response to Tpo alone contained MKs, and 71% of these were pure MK clones. However, 50% of clones formed in response to Tpo alone did not contain MKs, as examined by AchE staining. More than 10 MKs were detected in one third of the Tpo-recruited clones that were AchE-positive, but no clones contained more than 50 MKs. Interestingly, although synergy was observed with regard to number of clones formed when Tpo was combined with FL, FL+IL-11, or FL+IL-12, no increase in MK-containing clones or in the number of MKs per colony was observed when compared with those values for Tpo alone (Table 2). In contrast, SCF, which alone did not stimulate the formation of any MK-containing clones (data not shown), enhanced in combination with Tpo the number
of such clones almost threefold when compared with that for Tpo alone ($P < .05$). In addition, whereas Tpo alone did not stimulate formation of any colonies with more than 50 Mks, one in three of those formed in response to SCF+Tpo had greater than 50 Mks. In addition, unlike those clones formed in response to Tpo alone, almost all of the SCF+Tpo-recruited clones with Mks also contained other cell types. The addition of FL to SCF+Tpo resulted in the formation of significantly more MK-containing colonies, which all contained other cell types as well (Table 2).

Next, we examined the morphology of individual Giemsa-stained colonies (covering >10% of the well) formed in response to FL+SCF+Tpo from Lin-Sca-1+ cells plated at 1 cell/well in serum-depleted medium. These studies, in which 21 to 32 colonies in each of four separate experiments were analyzed, showed the presence of a low number of colonies containing exclusively undifferentiated blast (Bl) cells (7% ± 4%); mixed colonies containing Bl, macrophages (M), and granulocytes (G; 22% ± 5%); and Mks/Bl colonies (12% ± 3%) but showed mostly mixed Mks/Bl/M/G colonies (59% ± 2%). The same frequency of Mks-containing colonies was observed when using the AchE assay (data not shown). In contrast, FL+SCF-stimulated colonies contained no Mks (data not shown). Of the colonies containing Mks, 62% contained 1 to 49 Mks, 35% contained 50 to 200 Mks, and 4% contained more than 200 Mks, underscoring the potent ability of these progenitors to produce Mks in response to Tpo in combination with early acting cytokines. Thus, as much as 71% of the FL+SCF+Tpo-induced colonies covered more than 10% of the well contained Mks, most of which contained other cell lineages as well, thus showing that Tpo can stimulate the growth of Lin-Sca-1+ progenitors with the ability to produce multiple cell lineages.

Although the single cell studies described above showed that Tpo could enhance the growth of progenitors capable of producing multiple myeloid cell lineages, they did not establish to what degree Tpo-responsive Lin-Sca-1+ progenitors also had erythroid potential. This aspect was of particular interest because it recently has been shown that Tpo in combination with Epo can enhance the growth of committed erythroid progenitor cells. Thus, we next investigated the ability of Tpo to affect SCF-stimulated clonal growth of Lin-Sca-1+ progenitor cells cultured in methylcellulose in the presence of Epo (Table 3). Of 500 Lin-Sca-1+ cells, 33 produced colonies in response to SCF, which increased to 59 ($P < .05$) on addition of Tpo. Whereas most of the colonies formed in response to SCF alone were small, more...
Tpo promotes growth of putative stem cells

than 90% of the colonies formed in response to SCF+Tpo were greater than 0.25 mm in diameter. In agreement with previous studies, no pure erythroid colonies were formed, showing that Lin Sca-1+ progenitors are depleted in BFU-E.20,27 Although the frequency of erythroid-containing colonies was unchanged on the addition of Tpo to SCF-stimulated cultures, the absolute number of colonies with erythroid elements increased from 12 to 24. Moreover, the absolute number of colonies containing Mks (as well as other cell lineages) was potently enhanced in SCF+Tpo-stimulated cultures, from 8 to 47, as compared with that for SCF alone. Interestingly, the frequency and absolute number of multilineage GEMMk colonies increased more than threefold and sevenfold, respectively, when Tpo was combined with SCF, whereas a compensatory reduction was observed in the frequencies of GM and GEM colonies (Table 3).

Although it appeared that Tpo when acting in synergy with other cytokines promoted the growth of multipotent progenitor cells, it remained possible that most of the Lin- Sca-1+ progenitors growing in response to Tpo alone were not multipotent but rather were committed to the Mk lineage. However, the very limited proliferation of these progenitors (all clones contained <50 cells), and the finding that a high fraction produced exclusively Mks in response to Tpo alone, might as well be due to the requirement for other cytokines to uncover their proliferative and multilineage potential. To address this issue, Lin Sca-1+ cells were cultured at a concentration of 1 cell/well for 96 hours in Tpo alone, at which time all Tpo-responsive clones were identified and pulsed with a potent cytokine cocktail (G-CSF+IL-3+SCF+Tpo). When compared with Tpo-responsive Lin Sca-1+ progenitors that continued to be incubated in Tpo only, the number of pure Mk clones dropped from 28% to only 12% of the total clones formed, and the number of Mk-mix clones increased from 24% to 59% (P < .05; see Fig 6). Interestingly, the total number of non-Mk-containing clones also dropped from 48% to 30% on the addition of the cocktail to the Tpo-responsive progenitors (P < .05). Most of the Mk-mix and non-Mk-containing colonies contained granulocytes and/or macrophages (data not shown). Finally, a dramatic increase in the size of the Tpo-responsive clones was observed on the addition of the cocktail both in the Mk-mix and non-Mk-containing clones (P < .05; see Fig 6), suggesting that most of the Lin Sca-1+ progenitors responsive to Tpo alone are multipotent and have a high proliferative potential.

In vivo reconstitution studies with single Lin Sca-1-/Lin- Sca-1-Thy-1+ progenitor cells have shown that, although most of these are short-term- rather than long-term-reconstituting cells, they both represent predominantly multipotent progenitors capable of producing all hematopoietic lineages.38,39 Accordingly, it has been postulated that the restricted lineage expression often observed of these progenitors in in vitro cultures is a result of the culture conditions selectively promoting differentiation along specific lineages, usually granulocytic and monocytic,37 or, alternatively, is a consequence of interlineage competition.40 As a result of this, one might underestimate the number of multilineage progenitors in a specific population of primitive progenitor cells. However, because Lin Sca-1+ progenitor cells usually first commit to restricted lineages after prolonged culture, the potential for a specific lineage can also be determined by replating in a secondary culture supplemented with a lineage-selective cytokine combination, as previously shown for lymphohematopoietic progenitor cells by Hirayama et al.41 A total of 25 SCF+Tpo-induced colonies (in each of three separate experiments) were individually picked after 9 to 11 days of incubation and were replated in methylcellulose containing SCF+Epo. After such replating, 71% ± 5% of the primary SCF+Tpo-induced colonies formed BFU-E, showing that a large fraction of the Lin Sca-1+ progenitors responsive to SCF+Tpo, in addition to having a granulocytemacrophage and Mk potential, can produce erythroid cells as well. Next, we determined the morphology of the progeny of Lin Sca-1+ cells formed in response to Tpo alone and in combination with other cytokines in suspension culture (Fig 7). Whereas only 1 to 2 and 11 cells were formed per Lin Sca-1+ cell plated in response to Tpo and SCF, respectively, the combined action of Tpo+SCF potently increased the number of cells to 522 per Lin Sca-1+ cell plated. Few or

![Fig 6. Proliferative and multilineage potential of Lin Sca-1+ progenitors responsive to Tpo alone. Lin Sca-1+ cells were plated at 1 cell/well in serum-depleted medium as described in Materials and Methods. Cultures were stimulated with Tpo alone, and, after 96 hours of incubation, Tpo-responsive clones (mean of 25 ± 2 of 600 Lin Sca-1+ cells plated in each of three separate experiments) were identified and marked. Half of the clones were incubated for an additional 8 days in Tpo alone, whereas the rest were stimulated with predetermined optimal concentrations of G-CSF, SCF, IL-3, and Tpo (cocktail). Wells were scored for cell growth and stained with AChE (see Materials and Methods) or were transferred to slides in a cytospin centrifuge, fixed, and Giemsa-stained after 12 days of incubation at 37°C and 5% CO2 in air. Large colonies were defined as colonies covering more than 10% of well. Clones containing other cells in addition to Mks were scored as Mk-mix clones, whereas clones containing only Mks or no Mks were classified as Mk and non-Mk clones, respectively. Results represent mean percentages (±SEM) of three separate experiments. * P < .05 when compared with Tpo alone.](https://www.bloodjournal.org/content/4487.full)
Fig 7. Effects of Tpo on differentiation of Lin-Sca-1+ cells in suspension culture. Lin-Sca-1+ cells were cultured in the presence of predetermined optimal concentrations of cytokines as indicated in 500 μL serum-depleted medium. After 12 days of culture, total cell numbers were counted, and cytospin preparations of each group were analyzed with regard to Mk content in each experiment. Results represent means ±SEM of three separate experiments.

no viable cells were observed after 12 days of incubation in response to FL alone (data not shown). However, FL+Tpo synergistically increased the cell production from 1 to 2 cells (Tpo alone) to 23 cells per Lin-Sca-1+ cell plated. With regard to Mk production, SCF+Tpo potently synergized to induce the formation of 23 Mks per Lin-Sca-1+ cell plated, whereas only 0.2 and 0.06 Mks per Lin"Sca-1" cell plated were produced in response to Tpo and SCF, respectively. In contrast to the effect of SCF, FL did not increase Tpo-induced Mk production from Lin 'Sca-1" cells above that observed in response to Tpo alone. In addition to Mks, SCF- and SCF+Tpo-stimulated cultures contained a high proportion of monocytes and some blast cells, whereas mostly blast cells and some monocytes were formed in response to Tpo and FL+Tpo (data not shown).

Mk ploidy analysis. To determine whether Tpo in combination with SCF was capable of stimulating the formation of high-ploidy Mks from primitive progenitors in vitro, Lin"Sca-1" cells were cultured in suspension at 2,000 cells/mL in serum-depleted medium supplemented with SCF+Tpo. After 9 days of incubation, the cultured cells were double-stained with the 4A5 Mk-specific antibody30 and propidium iodide and were analyzed by flow cytometry (Fig 8, top). 4A5+ cells (Mk) had up to 128N ploidy, with the majority being of 16N and 32N ploidy (Table 4 and Fig 8). Analysis of SCF+Tpo-stimulated Lin 'Sca-1" cells on day 11 of culture showed a similar ploidy distribution as that observed on day 9 (data not shown). FL+SCF+Tpo-stimulated Lin 'Sca-1" cells contained 4A5+ Mks with a similar ploidy distribution as those stimulated by SCF+Tpo (Fig 8, bottom). Thus, Tpo in combination with SCF potently stimulates the production of high-ploidy Mks from multipotent Lin 'Sca-1" progenitor cells.

IL-3 has been implicated as potentially an important stimulator of Mk production in combination with Tpo.19 Although IL-3+Tpo potently stimulated Mk production from Lin 'Sca-1" progenitor cells, the ploidy of these Mks was significantly lower than that of those produced in response to SCF+Tpo (Table 4). Furthermore, Mks produced in response to IL-3+SCF had lower ploidy than that of those produced in response to IL-3+Tpo. Specifically, only 1% of Mks produced in response to IL-3+SCF were ≥32N, whereas 10% of Mks formed by IL-3+Tpo were ≥32N (P < .05). Thus, Tpo appears to be essential for optimal production of high-ploidy Mks from Lin 'Sca-1" progenitor cells.

Distinct effects of Tpo on the growth of Rhlo and Rhhi subsets of Lin 'Sca-1" progenitor cells. Previous studies have clearly established that the Rhlo subfraction of Lin 'Sca-1" BM cells contain all the long-term-reconstituting stem cells, whereas the Rhhi subfraction contains multipotent progenitors important for short-term reconstitution.24,26,42 In addition, the Rhlo subfraction in general requires stimulation by more cytokines for optimal growth than does the Lin 'Sca-1"Rhhi cells, and, accordingly, synergistic recruitment is most pronounced on Rhhi cells.24,42 Thus, we compared the ability of Tpo alone and in combination with SCF to stimulate the clonal growth of Rhlo and Rhhi Lin 'Sca-1" cells in serum-depleted medium (Table 5). In agreement with previous studies, SCF alone stimulated little clonal growth of Rhlo
Tpo promotes growth of putative stem cells

Table 4. Effects of SCF, IL-3, and Tpo on Ploidy Distribution of Mks Produced From Lin−Sca−1+ Progenitor Cells

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Ploidy, % of Total Mks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2N</td>
</tr>
<tr>
<td>SCF+Tpo</td>
<td>39(2)</td>
</tr>
<tr>
<td>IL-3+Tpo</td>
<td>42(5)*</td>
</tr>
<tr>
<td>IL-3+SCF</td>
<td>6(2)</td>
</tr>
</tbody>
</table>
| Lin−Sca−1+Mks were cultured in suspension in serum-depleted medium supplemented with IL-3+SCF (1,000 cells/mL), IL-3+Tpo (4,000 cells/mL), and SCF+Tpo (2,000 cells/mL). After 9 to 10 days of incubation 4A5+ cells were analyzed for ploidy distribution (propidium iodide uptake) as described in Materials and Methods and Fig 8. Results represent the means (±SEM) of three separate experiments. A Student’s unpaired t-test was performed comparing the ploidy distribution of IL-3+SCF- and IL-3+Tpo-stimulated cultures with those stimulated by SCF+Tpo. * P < .05. † P < .005.

Table 5. The Effect of Tpo on Clonal Growth of Lin−Sca−1+Rhhi Progenitor Cells

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Cells</th>
<th>Clusters (&lt;50 cells)</th>
<th>Colonies (&gt;50 cells)</th>
<th>Total Clones/300 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCF</td>
<td>Rh0°</td>
<td>4 (0)</td>
<td>0 (0)</td>
<td>4 (0)</td>
</tr>
<tr>
<td>Tpo</td>
<td>Rh0°</td>
<td>10 (1)</td>
<td>1 (1)</td>
<td>10 (0)</td>
</tr>
<tr>
<td>Tpo+SCF</td>
<td>Rh0°</td>
<td>12 (3)</td>
<td>16 (3)</td>
<td>27 (3)</td>
</tr>
<tr>
<td>SCF</td>
<td>Rh0°</td>
<td>21 (6)</td>
<td>0 (0)</td>
<td>21 (6)</td>
</tr>
<tr>
<td>Tpo</td>
<td>Rh0°</td>
<td>8 (2)</td>
<td>0 (0)</td>
<td>8 (2)</td>
</tr>
<tr>
<td>Tpo+SCF</td>
<td>Rh0°</td>
<td>17 (8)</td>
<td>9 (5)</td>
<td>26 (13)</td>
</tr>
</tbody>
</table>

Lin−Sca−1+Rh0° and Lin−Sca−1+Rhhi BM cells were isolated as described in Materials and Methods and plated at 1 cell/well in 20 μL x-vivo 15 supplemented with 1% BSA and predetermined optimal concentrations of cytokines (see Materials and Methods). Wells were scored for cluster and colony formation after 10 to 12 days of incubation at 37°C and 5% CO2 in humidified air.

DISCUSSION

Tpo has been shown to be a critical stimulator of megakaryocytogenesis and platelet production in vivo,12 which correlates well with its potent ability to stimulate in vitro growth and maturation of progenitor cells committed to the Mk lineage.1,16-19 Multiple findings have suggested a common origin of cells of the erythroid and megakaryocytic lineages, and Epo has been shown to stimulate Mk development in vitro.16,43-45 In line with these findings, it was recently shown that Tpo also can stimulate the growth and expansion of erythroid progenitor cells in combination with Epo,35,36 whereas several studies have found that Tpo does not affect the growth of committed granulocyte/macrophage progenitor cells in human or murine BM.13,18,19 In contrast, the ability of Tpo to affect the growth of primitive multipotent hematopoietic progenitor cells has not been investigated in detail. In a previous study, Zeigler et al36 showed that 50% of purified primitive (AA4-Sca−1+) murine fetal liver progenitor cells expressed c-mpl, and that an undefined fraction of these could proliferate and differentiate towards Mks in response to Tpo. However, the growth response to Tpo of these primitive high-proliferative potential (HPP) progenitors appeared very restricted, because less than 100, 600, and 300 Mks were produced from 10,000 progenitors in suspension culture in response to Tpo alone, Tpo+SCF, and Tpo+IL-3, respectively. The Mk production of these progenitors in response to Tpo was somewhat enhanced when cultured on fetal liver stroma cells,20 but, because the stroma potently stimulated proliferation in the absence of Tpo, the effects of Tpo observed in these cultures might have been on more committed progenitors formed in response to the growth-promoting activity of the stroma alone. More importantly, no clonogenic assays were performed allowing no conclusions to be made with regard to whether or not Tpo can stimulate the growth of multipotent progenitor cells.

In the present studies, Tpo alone stimulated clonal growth of a small fraction of Lin−Sca−1+ progenitor cells. Studies using subsequent addition of SCF+IL-3+G-CSF+Tpo showed that almost all of the progenitors responsive to Tpo alone had multilineage potential with an HPP rather than representing a subpopulation of primitive progenitors committed to the Mk cell lineage. Single cell experiments and delayed addition studies showed that the growth-stimulatory effect of Tpo is directly mediated on the progenitors and does not require the interaction with accessory cells.

A number of cytokines have been identified as potent stimulators of the growth of primitive hematopoietic progenitor cells.24 Most of these early acting cytokines have little or no ability to stimulate growth when acting individually, but, in combination with other cytokines such as the CSFs, they can enhance growth in a synergistic manner.24 Tpo synergized with many of these early acting cytokines (eg, FL, SCF, and IL-11, but not IL-12) to enhance the growth of Lin−Sca−1+ progenitors. This synergy was observed both as a recruitment of progenitors that were unresponsive to single cytokines (increased clonal numbers) and as an enhanced proliferation of the individual progenitors (enhanced clonal size).
Unlike its ability to synergize with FL and SCF, Tpo did not synergize with G-CSF or GM-CSF to enhance the clonal growth of Lin" Sca-1" progenitors. When Tpo was combined with IL-3, a slightly more than additive number of clones were formed, and a synergistic increase in the number of colonies (> 50 cells) was observed. In sharp contrast to Tpo, Epo had no ability to stimulate the growth of Lin" Sca-1" progenitors alone or in combination with SCF or FL. In addition, Tpo and Epo did not synergize to enhance the growth of Lin" Sca-1" progenitors, in contrast to their ability to synergize on more committed progenitors. Thus, despite the corresponding roles of Tpo and Epo as critical and selective regulators of megakaryocytopoiesis and erythropoiesis, respectively, Tpo, but not Epo, appears to affect the growth of primitive multipotent progenitor cells. Therefore, it appears likely that, whereas c-mpl is expressed on primitive multipotent progenitors, expression of the Epo receptor is restricted to progenitors committed to the erythroid and megakaryocytic lineages.

Flt3 and c-kit and their corresponding ligands (FL and SCF, respectively) have been shown to be key regulators of early hematopoiesis54,55 and to potently stimulate the growth of primitive progenitors in combination with multiple other cytokines. Thus, the potential ability of Tpo to interact with these two cytokines was of particular interest. In addition to synergizing with FL and SCF individually, Tpo also enhanced the number of clones formed from Lin" Sca-1" progenitor cells when combined with both FL and SCF. In particular, Tpo acted to enhance the number of large colonies formed in response to FL+SCF. Thus, although FL and SCF have largely overlapping activities with regard to stimulating growth of primitive progenitors, the results of the present study support those of previous studies suggesting that the two cytokines can interact in a complimentary fashion.51,55

Whereas SCF when combined with Tpo enhanced the number of Mk-containing colonies almost threefold as well as the number of Mks per colony, FL did not increase the number of MK-containing colonies or the number of Mks per colony. The reason for this dramatic difference between SCF and FL might be due to a more restricted expression pattern for Flt3 than c-kit. Whereas Flt3 expression in the hematopoietic system appears to be quite restricted to primitive multipotential or lymphoid progenitors, c-kit shows a wider expression and has also been shown to be expressed on myeloid and erythroid progenitors.54,55 Other recent studies indicate that SCF can synergize with Tpo to recruit committed Mk progenitors as well.18,17 Therefore, it seems possible that the progenitors recruited by Tpo+SCF or Tpo+FL might require prolonged stimulation by two cytokines to proceed to Mk-producing progenitors, and that Flt3 but not c-kit expression is lost before responsiveness to Tpo alone is obtained. Thus, SCF in combination with Tpo might be critically involved in stimulating megakaryocytopoiesis from multipotent progenitor cells. In line with this, all Tpo-responsive progenitors express c-kit (Ramsfjell and Jacobsen, unpublished observations, September 1995).

Murine Lin" Sca-1" BM cells have been shown to be highly enriched in primitive multipotent progenitor cells capable of short-term as well as long-term multilineage reconstitution.18,22,23,28-36,39 Although constituting only 0.1% to 0.2% of the BM cells, they contain all the long-term-repopulating cells. In addition, this population is depleted in lineage-committed progenitors and consists predominantly of multipotent progenitor cells requiring multiple cytokines for optimal growth in vitro, whereas only SCF and IL-3 can induce limited growth as single cytokines.44,47,56 However, as any enriched stem cell population, Lin" Sca-1" progenitor cells are heterogeneous, and, although they consist predominantly of multipotent progenitor cells, most of these have a short-term—rather than long-term—reconstituting ability.12,23,38,39 The Lin" Sca-1" cells can be somewhat more enriched for stem cell activity by sorting the c-kit" subfraction.42 However, because all Lin" Sca-1" stem cells as well as clonogenic progenitors are c-kit", it is clear that the clonogenic progenitors investigated here are Lin" Sca-1" c-kit". Long-term—repopulating activity can also be enhanced by sorting for Thy-1 (twofold),43,42 by isolating the Rhb fraction as done in the present study (fourfold),34,26 or by treating cells with 5-fluorouracil.55 Alternatively, stem cell populations can be highly enriched by other methods.1,51,52 However, no method has yet been able to purify a homogenous population of long-term—repopulating stem cells, and, accordingly, firm conclusions regarding growth regulation of the pluripotent long-term—reconstituting stem cells cannot be drawn from in vitro studies such as those described here, although there are increasingly more studies suggesting that the long-term—reconstituting stem cells can also proliferate extensively in short-term in vitro cultures in response to defined cytokines.54,55 Regardless, the Lin" Sca-1" and Lin" Sca-1" Rhb BM progenitor cell populations used here are well characterized and suitable for studies of early multipotent progenitor cells.34,38,42,54 That Tpo does stimulate the growth of multipotent progenitor cells was supported by the finding that most Mk-containing colonies produced in response to SCF+Tpo or FL+SCF+Tpo contained other myeloid cell lineages as well, and, in methylcellulose cultures containing Epo, Tpo in combination with SCF potently enhanced multilineage (GEMMk) colony formation. Thus, although the effects of Tpo administration in vivo and studies of peripheral blood in c-mpl—deficient mice clearly show that Tpo is a lineage-selective cytokine under steady-state conditions,17 the present studies support that it is lineage-specific.

The clinical relevance of the present findings is supported by recent studies showing that Tpo in myeloaclated mice and nonhuman primates in addition to promoting platelet recovery also accelerates the recovery of red blood cells and neutrophils.55,56 In addition, the present findings might have physiological significance because a recent study showed that the number of hematopoietic progenitors of multiple lineages and the number multipotent progenitors are reduced in c-mpl—deficient mice, suggesting that Tpo might play a role in maintaining primitive hematopoietic progenitor cells.57

Although Lin" Sca-1" progenitors recruited by Tpo were predominantly multipotent, the unique ability of these HPP Tpo-responsive progenitors to produce large numbers of high-ploidy Mks was underscored by the finding that some
TPO PROMOTES GROWTH OF PUTATIVE STEM CELLS

individual Lin- Sca-1+ progenitors (ie, stimulated by FL+SCF+Tpo) produced more than 200 Mks, and that 38% of the Mk-producing HPP colonies contained more than 50 Mks. The present observation that SCF+Tpo > IL-3+Tpo > IL-3+SCF with regard to stimulating production of Mks with high ploidy might potentially have implications as to the relative role of these cytokines in regulating Mk ploidy. The difference between the very efficient production of Mks in response to SCF+Tpo in the present studies (>23 Mks per Lin- Sca-1+ cell plated) as compared with the marginal Mk production in the studies of Zeigler et al4 (0.06 Mks per AA4+Sca-1+ fetal liver cell) is most likely (at least in part) due to the fact that we used a serum-depleted medium, whereas Zeigler et al40 used an FCS-supplemented medium to study Mk production. When using an FCS-supplemented medium, we also found a very restricted ability of Lin-Sca-1+ BM cells to produce Mks in response to SCF+Tpo (Ramsfjell and Jacobsen, unpublished observation, September 1995).

Lin- Sca-1+ progenitors were further fractionated into Rhb cells, shown to contain all long-term-reconstituting cells, and Rhb cells, thought to be important for short-term multilineage reconstitution.24-26,42 Interestingly, in agreement with the results of previous studies, whereas almost all progenitors responsive to SCF alone were in the Rhb fraction,24 the ability of SCF and Tpo in combination to synergistically recruit an increased number of Lin- Sca-1+ progenitors to proliferate was exclusively observed on the Rhb cells. However, the ability of SCF and Tpo to synergistically enhance the proliferation of individual progenitors was observed in both populations. These findings further support that Tpo can stimulate the growth of a primitive set of multipotent progenitors, although it still remains to be determined whether it acts on the long-term-repopulating stem cells.

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Thrombopoietin, but not erythropoietin, directly stimulates multilineage growth of primitive murine bone marrow progenitor cells in synergy with early acting cytokines: distinct interactions with the ligands for c-kit and FLT3

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