In this study, we explored whether thrombopoietin (Tpo) has a direct in vitro effect on the proliferation and differentiation of long-term repopulating hematopoietic stem cells (LTR-HSC). We previously reported a cell separation method that uses the fluorescence-activated cell sorter selection of low Hoescht 33342/low Rhodamine 123 (low Ho/low Rh) fluorescence cell fractions that are highly enriched for LTR-HSC and can reconstitute lethally irradiated recipients with fewer than 20 cells. Low Ho/low Rh cells alone had high proliferative potential in vitro in the presence of stem cell factor (SCF) + interleukin-3 (IL-3) + IL-6 (90% to 100% HPP-CFC). Tpo alone did not induce proliferation of these low Ho/low Rh cells. However, in combination with IL-3, Tpo had several synergistic effects on cell proliferation. When Tpo was added to single growth factors (either SCF or IL-3 or the combination of both), the time required for the first cell division of low Ho/low Rh cells was significantly shortened and their cloning efficiency increased substantially. Moreover, the subsequent clonal expansion at the early time points of culture was significantly augmented by Tpo. Low Ho/low Rh cells, when assayed in agar directly after sorting, did not form megakaryocyte colonies in any growth condition tested. Several days of culture in the presence of multiple cytokines were required to obtain colony-forming units-megakaryocyte (CFU-Mk). In contrast, more differentiated, low Ho/high Rh cells, previously shown to contain short-term repopulating hematopoietic stem cells (STR-HSC), were able to form megakaryocyte colonies in agar when cultured in Tpo alone directly after sorting. These data establish that Tpo acts directly on primitive hematopoietic stem cells selected using the Ho/Rh method, but this effect is dependent on the presence of pluripotent cytokines. These cells subsequently differentiate into CFU-Mk, which are capable of responding to Tpo alone. Together with the results of previous reports of its effects on erythroid progenitors, these results suggest that the effects of Tpo on hematopoiesis are greater than initially anticipated.

© 1996 by The American Society of Hematology.

The Effect of Thrombopoietin on the Proliferation and Differentiation of Murine Hematopoietic Stem Cells

By Ewa Sitnicka, Nancy Lin, Gregory V. Priestley, Norma Fox, Virginia C. Broudy, Norman S. Wolf, and Kenneth Kaushansky

The Hypothesis that circulating platelet levels and megakaryocytopoiesis are controlled by a humoral regulator was proposed by Yamamoto1 and named thrombopoietin (Tpo) by Kelemen et al. in 1958. Since that time, many groups have attempted to isolate and purify Tpo. A major step in understanding the regulation of megakaryocytopoiesis was made with the recent identification of the c-mpl protooncogene. Ten years ago, Wendling et al. described a transforming viral complex that induces a myeloproliferative syndrome in mice. The transforming gene v-mpl was subsequently identified by Souryi et al. and the cellular homologue c-mpl was cloned from human HEL cells in 1992. Sequence analysis of human2 and murine3 c-mpl showed that the encoded protein was likely a novel member of the hematopoietic receptor family. However, its ligand was unknown.

Using different strategies, several groups have recently purified the ligand for c-Mpl or have obtained cDNA by functional expression cloning. Subsequent characterization of the recombinant protein quickly established that the ligand for c-Mpl is identical to Tpo. Treatment of mice with Tpo substantially increased platelet counts within 5 days due to increased numbers of marrow megakaryocytes and their progenitor cells. In vitro, Tpo was shown to stimulate the growth of murine megakaryocyte colony-forming cells5,6 and to increase megakaryocyte ploidy. Thus, Tpo appeared initially to act as a lineage-specific late-acting hematopoietic growth factor, much like the effects of erythropoietin (Epo) and granulocyte colony-stimulating factor (G-CSF) on erythropoiesis and myelopoiesis, respectively. More recent studies have called this initial conclusion into question. Several groups have now shown that myelopoiesis and erythropoiesis are also affected by Tpo. Tpo increased the numbers of colony-forming units–granulocyte-macrophage (CFU-GM)7 and burst-forming units-erythroid (BFU-E)8 in vivo and lead to a redistribution of colony-forming units-erythroid (CFU-E) from marrow to spleen. Moreover, Tpo acted in synergy with Epo to increase the growth of BFU-E and the generation of CFU-E from marrow cells.17,18 These findings suggest that Tpo also acts on other hematopoietic cell types. To further investigate this finding, we have studied whether Tpo exerts a stimulatory effect on primitive hematopoietic stem cells. Using a previously reported cell separation method, based on the selection of cell fractions that retain very low levels of Hoescht 33342 and Rhodamine 123 dye (low Ho/low Rh), a cell population highly enriched for long-term repopulating hematopoietic stem cells (LTR-HSC) was selected. We found that although Tpo alone did not induce proliferation of low Ho/low Rh cells, in synergy with interleukin-3 (IL-3), stem cell factor (SCF), or both, Tpo acted to accelerate their entry into cell cycle and to stimulate their immediate progeny to proliferate to a greater degree than in its absence. In contrast, Tpo alone could directly stimulate the proliferation of the more-differentiated low Ho/high Rh cells. Because this latter cell fraction contains short-term repopulating hematopoietic stem cells (STR-HSC), it appears that Tpo acts on cells before the stage of lineage commitment. These results suggest that Tpo acts on lineage-committed and immature cells and that the
therapeutic response to its administration may be greater than previously anticipated.

MATERIALS AND METHODS

Animals. In all experiments, male and female F1 hybrids from the cross C57Bl/6 × DBA/2 were used. Three- to 6-month-old mice were obtained from our NIA-derived breeding colony, which is maintained under strict specific pathogen-free conditions and is routinely tested and confirmed to be free of all known mouse pathogens.

Bone marrow cells. Mice were killed and the femurs and tibias were removed aseptically and repetitively flushed with phosphate-buffered saline (PBS) with 10% citrate phosphate dextrose (CPD) solution, 1% fetal bovine serum (FBS; Biocel, Rancho Dominguez, CA), and 50 U/mL DNase (Sigma, St Louis, MO). This solution was used as collection medium and for cell resuspension through the lineage-depletion steps. The cell suspension was drawn three times through successively smaller bore needles and finally passed through a 100-mesh stainless steel screen to obtain a single-cell suspension. The low-density bone marrow cells were isolated on a gradient by layering 5-mL aliquots of 10^7 cells/mL over 3.0 mL Nycodenz (1.085 g/mL; Nycosomed, Oslo, Norway). Cells were centrifuged for 20 minutes at 400 g, collected from the interface, and washed twice with collection medium at 4°C.

Lineage-committed cell depletion and vital dye staining. During antibody incubation and magnetic bead depletion of lineage-committed precursors, cell suspensions were held at 4°C. Rat-antimouse monoclonal antibodies were used in a titrated mixture. Anti-YW25.12.7 (erythroid) was a gift from Dr Ivan Bertoncello (Peter MacCallum Cancer Institute, Melbourne, Australia). Anti-7/4 (neutrophils and activated macrophages) was purchased from Serotec (McCallum Cancer Institute, Melbourne, Australia). Anti-Gr-l (myeloid), anti-CD1 lb (anti-Terl9; erythroid), anti-Gr-l (myeloid), and anti-CD11b (anti-Mac-1) were purchased from PharMingen (San Diego, CA). A total of 10^7 cells/mL were incubated with an equal volume of the antibody mixture for 30 minutes at 4°C, washed over a 1-mL FBS, and placed into the Dynal magnet. Cells not bound with the magnetic beads remained in suspension and were carefully removed using Pasteur pipette and this process was repeated with fresh beads (this fraction was designated lineage-negative cells). Cells were counted and resuspended in 10 µmol/L Hoechst 33342 (Ho; Sigma) in Hank’s Balanced Salt Solution (HBSS) with 20 mmol/L HEPES, 1 g/L glucose, 10% FBS; adjusted to pH 7.2 with NaHCO₃, and incubated at 37°C for 1 hour. During the last 20 minutes of the Ho incubation, Rhodamine 123 (Rh-123; Sigma) at 0.1 µg/mL final concentration was added to the cell suspension. After dye incubation, the cells were chilled to 4°C, washed twice with PBS + 1% FBS, resuspended in PBS + 1% FBS + propidium iodide (PI; 2 µg PI/mL; for detection of dead cells and residual viable neutrophilic promyelocytes/metamyelocytes), and sorted.

Fluorescence-activated cell sorter (FACS) selection of LTR-HSC–enriched cell fractions. Cell sorting of the lineage-depleted preparation was performed on an Ortho Cytofluorograph model 50H/2131 (Ortho Diagnostic System, Westwood, MA) equipped with two spatially separated argon-ion lasers (model 90-6 [Coherent Corp, Palo Alto, CA] and model 166-05 [Spectra Physics, Mountain View, CA]). Monochromatic light at 351 to 364 nm was used for Ho excitation and fluorescence emission detected at 420 nm using a long pass filter. Rh-123 excitation was at 488 nm and fluorescence emission was detected at 515 to 535 nm. After exclusion of PI-positive cells, previously published settings were used to successively select a 10% blast cell window based on high forward, low right angle scatter, and, sequentially, the lowest 3% to 5% of the Ho fluorescence histogram and then the lowest 15% of the Rh-123 fluorescence histogram. Sorted cells (henceforth designated low Ho/low Rh cells) were directly and individually deposited into 96-well plates containing the culture medium. In some experiments, as indicated, high Rh-123 fluorescence cells (highest 65% to 100% of Rh retention) were used; this cell fraction was designated low Ho/high Rh.

Single-cell culture conditions. Single sorted cells were cultured in 96-well U-bottomed plates (Corning) in Iscove’s modified Dul-

---

Table 1. Effect of Tpo on Cloning Efficiency and Clone Size of Low Ho/low Rh Cells Cultured as Single Cells

<table>
<thead>
<tr>
<th>Growth Factors Added</th>
<th>Cloning Efficiency*</th>
<th>% Macroclones (&gt;100,000 cells) on day 14</th>
<th>Range of Clone Size on day 14 (cell no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
<td>Exp 1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tpo</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>SCF</td>
<td>18</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>SCF + Tpo</td>
<td>88</td>
<td>93</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>26</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>IL-3 + Tpo</td>
<td>44</td>
<td>70</td>
<td>6</td>
</tr>
<tr>
<td>SCF + IL-3</td>
<td>94</td>
<td>96</td>
<td>53</td>
</tr>
<tr>
<td>SCF + IL-3 + Tpo</td>
<td>98</td>
<td>100</td>
<td>67</td>
</tr>
<tr>
<td>SCF + IL-3 + IL-6</td>
<td>97</td>
<td>99</td>
<td>90</td>
</tr>
<tr>
<td>SCF + IL-3 + IL-6 + Tpo</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Low Ho/low Rh cells were individually deposited from the cell sorter in 96-well U-bottomed plates containing culture medium (see the Materials and Methods) and growth factors as follows: 115 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL IL-11, 50 ng/mL SCF, and 0.5 ng/mL Tpo. Single cytokines were used at the following concentrations: IL-3 at 480 ng/mL, SCF at 50 ng/mL, and Tpo at 0.5 ng/mL. Data represent values from two independent experiments; 20 to 50 clones in each group were analyzed. Cells were cultured for 14 days, and at day 14 clone size and cell morphology were also analyzed.

* Percentage of single cells that divided at least once.
becco’s medium (IMDM; GIBCO BRL, Grand Island, NY) medium supplemented with cytokines and with 12.5% FBS, 12.5% horse serum (HS; GIBCO), 2 × 10^{-7} mol/L 2-mercaptoethanol (2-ME; Sigma), 10^{-7} mol/L hydrocortisone (HC; Sigma), antibiotics (penicillin/streptomycin; GIBCO), and various combinations of IL-3, IL-6, IL-11, SCF, and Tpo. Different lots of FBS and HS used in cultures were selected for their ability to support HFP colony formation of lineage-negative cells in agar. In all experiments, cells were cultured for 2 to 3 weeks. The use of U-bottom plates facilitated the settling of single cells to center-bottom, which allowed direct observation. Each well was checked for the presence of a single cell, mapped, and then observed for the culture period. Subsequently, clones ranging from 2 to 64 cells could be directly enumerated. The number of cells per well were directly counted using a phase contrast inverted microscope at 200× magnification. At 14 days of the culture, the clone size was determined by hemacytometer counts. Cell viability was determined by trypan blue exclusion. Cell morphology was estimated on slides stained by the Giemsa-Wright method. In some experiments, as indicated, sorted cells were cultured at 100 cells per well in 24-well plates in the presence of IL-3 and IL-6 and SCF with or without Tpo for different periods of time and then washed, counted, and assayed for megakaryocyte colony formation in the presence of IL-3 + IL-11 + SCF + Tpo.

**Colony-forming unit-megakaryocyte (CFU-Mk) assay.** Bone marrow cell fractions were plated in IMDM (GIBCO) supplemented with various combinations of IL-3, IL-11, SCF, and Tpo; 10% HS (Hyclone, Logan, UT); 5 × 10^{-7} mol/L 2-mercaptoethanol (Sigma); and penicillin-streptomycin (Sigma). They were then made semisolid with 0.275% agar (Difco, Detroit, MI) in triplicate 1-mL plates. Different bone marrow fractions were plated at different cell concentrations. Lineage-negative cells were plated at 1 × 10^5 cells/plate, sorted low Ho/low Rh cells at 100 to 300 cells per dish, and sorted low Ho/high Rh cells at 300 to 500 cells per dish. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂, and megakaryocytic colonies were counted on day 5 using an inverted microscope. Megakaryocyte colonies were defined as containing at least three large refractive cells.

**Sources of cytokines.** Purified, recombinant growth factors were kindly and generously provided as follows: rat SCF and mouse IL-3 from Amgen Inc (Thousand Oaks, CA); human IL-6 (rhuIL-6) from Dr Douglas Williams (Immunex Corp, Seattle, WA); and mouse IL-11 (rmIL-11) from Drs Paul Schendel and Stanley Wolf (Genetics
EFFECTS OF Tpo ON HEMATOPOIETIC STEM CELLS

Institute, Boston, MA). Recombinant murine Tpo was expressed in baby hamster kidney (BHK) cells as previously described.14

The concentrations of growth factors were as follows: 100 ng/mL IL-3, 50 ng/mL SCF, 20 ng/mL IL-6, 20 ng/mL IL-11, and 0.5 ng/mL Tpo.

RESULTS

Effect of Tpo on the proliferation of LTR-HSC. To examine the direct effect of Tpo on LTR-HSC, sorted low Ho/low Rh cells were cultured using a single-cell culture system in the presence of different concentrations of growth factors. This approach allowed us to measure the kinetics of proliferation, the time required to undergo the first cell division, and the proliferative potential. As shown in the Table 1, Tpo alone had a minimal effect on the proliferation of low Ho/low Rh cells. Only a small proportion of these cells (12% ± 8%) were able to divide in the presence of Tpo alone, and these clones went through only 1 to 2 cell divisions during 14 days in culture. In contrast, when Tpo was added to SCF, the number of proliferating low Ho/low Rh cells increased from 32% ± 20% in SCF alone to 88% ± 7% with Tpo, and the addition of Tpo allowed these clones to undergo more cell divisions during 14 days of the culture (Table 1). Tpo also had a synergistic effect in combination with IL-3. The number of dividing low Ho/low Rh cells was increased from 27% ± 2% to 57% ± 18% when Tpo was present; however, the total number of cells in the clones after 14 days of culture was not changed.

The addition of Tpo to the multiple growth factor combinations that provided optimal growth stimulation, ie, IL-3 + IL-6 + SCF (Table 1) or IL-3 + IL-11 + SCF (data not shown), did not change either the number of proliferating low Ho/low Rh cells or the size of proliferating clones. However, even under these apparently optimal growth conditions,
the presence of Tpo augmented the rate of proliferation of LTR-HSC during the early period of the culture. As shown in Fig 1A through C, the presence of Tpo was associated with twofold greater cell numbers at 4 to 5 days after culture initiation under both suboptimal growth conditions (IL-3 or SCF alone) or in the presence of optimal concentration of cytokines (IL-3 + IL-6 + SCF).

**Effect of Tpo on the entry of LTR-HSC into cell cycle.** Previous studies with IL-11,22 IL-6,23,24 and G-CSF25 have shown that these cytokines can accelerate the entry of primitive hematopoietic progenitors into the cell cycle. To determine whether Tpo shares this property, we noted the day at which single plated low Ho/low Rh cells began to divide in response to different combinations of hematopoietic growth factors. When used with either SCF alone or IL-3 alone, Tpo accelerated the entry of low Ho/low Rh cells into cell cycle (Fig 2A and B). Cells plated in the presence of IL-3 plus SCF or of IL-3, IL-6, and SCF, without Tpo, also entered the cell cycle sooner when Tpo was present, although the results were less impressive (Fig 2C and D).

**Megakaryocyte colony formation by hematopoietic stem cells.** We next examined and compared the ability of different bone marrow cell populations to directly form megakaryocytic colonies in response to three growth factor conditions: IL-3 alone, Tpo alone, or SCF + IL-3 + IL-11 + Tpo. As shown in Fig 3, a bone marrow cell population depleted of lineage-committed cells (lineage-negative cells) formed megakaryocyte colonies in response to all growth conditions tested. However, the number of colonies formed in the presence of SCF + IL-3 + IL-11 + Tpo was significantly higher.
EFFECTS OF Tpo ON HEMATOPOIETIC STEM CELLS

Fig 3. CFU-Mk formation in the various bone marrow cell fractions. CFU-Mk formation was assayed directly by plating in agar (see the Materials and Methods) after sorting and in different growth factor combinations: IL-3 alone (II), Tpo alone (III), and IL-3 + IL-11 + SCF + Tpo (III). The data represent the mean colony numbers ± SD of triplicate plates from one to two similar experiments.

110 ± 13 per 10^5 compared with 16 ± 7 per 10^5 in IL-3 alone or 36 ± 7 per 10^5 in Tpo alone. When low Ho/low Rh (0% to 15% Rh) cells were assayed, no CFU-Mk growth was seen in any of the growth conditions tested (Fig 3).

Because our initial experiments indicated that Tpo augmented the proliferation of the primitive low Ho/low Rh cells, we performed a time course study to determine when the progeny of such cells developed into CFU-Mk. As shown in Fig 4, CFU-Mk were not detectable in significant numbers in suspension cultures of low Ho/low Rh cells until day 5, regardless of whether Tpo was present, but the numbers of CFU-Mk generated were approximately double that present in cultures initiated without Tpo. Consistent with these data, when low Ho/high Rh (65% to 100% Rh) cells were plated (such cells previously have been shown to be derived from low Ho/low Rh,26) the frequency of CFU-Mk-derived colonies increased substantially. Thus, the low Ho/high Rh fraction, a population enriched for short-term repopulating cells, contains high numbers of megakaryocyte progenitor cells, whereas the more primitive low Ho/low Rh cells were at a stage undetectable for colony formation.

DISCUSSION

Tpo is a major regulator of the proliferation, differentiation, and maturation of megakaryocytes. However, results from recent studies suggest that Tpo can act not only as a lineage-specific hematopoietic growth factor, but also on other hematopoietic cell types.17-19 To further investigate this hypothesis, we choose to study the effect of Tpo on the proliferation and differentiation of hematopoietic stem cells at two selected levels of development. Using a previously reported purification strategy based on the selection of cell fractions that retain very low levels of Hoechst 33342 and Rhodamine 123 dye, we selected two cell populations: low Ho/low Rh, enriched for long-term repopulating stem cells, and low Ho/high Rh, containing short-term repopulating stem cells.20 Both cell fractions were previously characterized in vivo and in vitro.20 To examine the direct effects of Tpo, a single-cell culture system was used. We found that, by itself, Tpo did not stimulate the proliferation of LTR-HSC present in the low Ho/low Rh cell fraction. Also, when plated in agar directly after sorting, low Ho/low Rh cells did not form CFU-Mk-derived colonies in any of the growth conditions tested. In contrast, STR-HSC in the low Ho/high Rh cell fraction proliferated and formed megakaryocyte colonies when plated in agar directly after sorting (at day 0), indicating that megakaryocyte progenitor cells were present in this bone marrow fraction. Suspension cultures of low Ho/low Rh population indicated that the generation of CFU-Mk takes about 5 days. However, when combined with IL-3 or SCF, Tpo stimulated proliferation of the low Ho/low Rh cells. This is in agreement with data reported in abstract form by Ku et al,27 although the target cell population used in that study was not characterized in vivo.

The effect of Tpo on the hematopoietic stem cell population was manifest in several ways. The time required for low Ho/low Rh cells to undergo their first cell division was shorter in the presence of IL-3 or SCF if Tpo was present. These results indicate that Tpo accelerates stem cell entry into the cell cycle in a manner similar to that previously reported for IL-6,23,24 IL-11,22 or G-CSF,25 and suggest that Tpo acts directly on the LTR-HSC selected using the Ho/low Rh cell fraction. Additional experiments gave similar results.
Rh method. Also, the number of descendent cells derived from single low Ho/low Rh cells in the presence of cytokine combinations with Tpo was increased compared with the cytokine combinations without it.

The hypothesis that Tpo may act directly on hematopoietic stem cell can be inferred from studies of Wendling et al47 working with the myeloproliferative leukemia virus (MPLV), which induces an acute myeloproliferative syndrome in mice. After infection, characteristic marrow findings include the induced proliferation and differentiation of multiple hematopoietic lineages, indicating the possibility, at least, that the hematopoietic stem cell population is the in vivo target for MPLV transformation.5,6,7,8 Because v-mpl represents a constitutively activated Tpo receptor, it follows that Tpo could affect the hematopoietic stem cell. More recently, studies have appeared reporting that human CD34+CD38- hematopoietic cells, a cell population highly enriched in hematopoietic stem cells, express c-Mpl.9,10 These results suggest that the Tpo receptor may be present on hematopoietic stem cells.

Our results clearly establish that Tpo acts directly on primitive hematopoietic stem cells selected using the Ho/Rh method, but that its effects are dependent on the presence of either IL-3 or SCF. Noncycling, G0 low Ho/low Rh cells enter the cell cycle sooner in the presence of Tpo and, once induced to begin to divide, undergo a greater number of cell divisions per unit time. Previous studies by our group and others have also shown that Tpo can act in synergy with Epo to affect erythropoiesis.11,12 Taken together, these results indicate that Tpo can affect multiple stages of hematopoiesis and suggest that its therapeutic efficacy may be greater than initially anticipated.

REFERENCES


27. Ku H, Kaushansky K, Ogawa M: Thrombopoietin, the ligand for MPL receptor, synergises with steel factor and other early-acting cytokines in supporting proliferation of primitive hematopoietic progenitors in mice. Blood 86: 256a, 1995 (abstr, suppl 1)


The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells

E Sitnicka, N Lin, GV Priestley, N Fox, VC Broudy, NS Wolf and K Kaushansky