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

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

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 cloned with 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.

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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 patho-
gens.*

Bone marrow cells. Mice were killed and the femurs and tibias
were removed aseptically and repetitively flushed of marrow with
phosphate-buffered saline (PBS) with 10% citrate phosphate dextrose
(CPD) solution, 1% fetal bovine serum (FBS; Biocell, Rancho
Dominguez, CA), and 50 U/mL DNase (Sigma, St Louis, MO).
This solution was used as collection medium and for cell resuspen-
dion 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 iso-
lated on a gradient by layering 5-mL aliquots of 10^7 cells/mL over
3.0 mL Nycodenz (1.085 g/mL; Nycosol, Oslo, Norway). Cells were
centrifuged at 20g for 3 minutes, resuspended, transferred
polypropylene tube, 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 re-
peated with fresh beads (this fraction was designated lineage-nega-
tive 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,
twice washed 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.

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>Range of Clone Size (on day 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%) Macroclones (&gt;100,000 cells) on day 14</td>
<td>Range of Clone Size (on day 14 (cell no.)</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tpo</td>
<td>18</td>
<td>2 to 4</td>
</tr>
<tr>
<td>SCF</td>
<td>36</td>
<td>2 to 100</td>
</tr>
<tr>
<td>SCF + Tpo</td>
<td>65</td>
<td>10^5 to 10^6</td>
</tr>
<tr>
<td>IL-3</td>
<td>6</td>
<td>2 to 4</td>
</tr>
<tr>
<td>IL-3 + Tpo</td>
<td>14</td>
<td>10^5 to 10^6</td>
</tr>
<tr>
<td>SCF + IL-3</td>
<td>70</td>
<td>10^5 to 10^6</td>
</tr>
<tr>
<td>SCF + IL-3 + Tpo</td>
<td>81</td>
<td>10^5 to 10^6</td>
</tr>
<tr>
<td>SCF + IL-3 + IL-6</td>
<td>94</td>
<td>10^5 to 10^6</td>
</tr>
<tr>
<td>SCF + IL-3 + IL-6 + Tpo</td>
<td>100</td>
<td>10^5 to 10^6</td>
</tr>
</tbody>
</table>

* Percentage of single cells that divided at least once.

Macrophages and Activated Macrophages was purchased from Serotec.
Terl19 (erythroid), anti-Gr-1 (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 twice with collection medium
at 4°C, and then rosetted twice with magnetic beads coated with sheep
anti-
Dias, CA), and an equal volume of 5X the number of magnetic beads
was added dropwise to the cell suspension and gently mixed with a pipette. The suspension was immediately centri-
fuged at 20g for 3 minutes, resuspended, transferred to a 5-mL
polystyrene tube, 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 re-
peated with fresh beads (this fraction was designated lineage-nega-
tive cells). Cells were counted and resuspended in 10 μmol/L
Hoechst 33342 (Ho; Sigma) in Hank's Balanced Salt Solution
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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,
twice washed 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.

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

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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 × 10−3 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 (Hylone, Logan, UT); 5 × 10−3 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 × 10⁵ 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.³⁶

**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 (mIL-11) from Drs Paul Schendel and Stanley Wolf (Genetics

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**Fig 1.** Effect of Tpo on clonal proliferation. Single low Ho/low Rh cells were cultured for 14 days under various conditions and the clone size was estimated at various time points. (A) (■) Cell number in cultures grown in presence of IL-3 + IL-6 + SCF; (■) cultures with these cytokines plus Tpo. (B) Cultures with IL-3 alone (■) and IL-3 plus Tpo (■). (C) Cultures with SCF alone (■) and SCF plus Tpo (■). The data in all three panel represent mean values ± SD from two independent experiments in which 50 to 60 clones were analyzed. * P < .01.
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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,
Fig 2 (cont’d). (C) The percentage of clones derived from single low Ho/low Rh cell dividing in response to (○) IL-3 + SCF or to (●) IL-3 + SCF + Tpo. The top panel and the bottom panel represent the data from two independent experiments. (D) The percentage of clones dividing in response to (○) IL-3 + IL-6 + SCF or to (●) IL-3 + IL-6 + SCF + Tpo. The top panel and the bottom panel represent the data from two independent experiments. In each experiment, 50 to 60 clones were analyzed. Results are expressed as a cumulative percentage.

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:

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

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 conditions tested. In contrast, STR-HSC in the low Hohigh 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, 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, IL-11, or G-CSF, and suggest that Tpo acts directly on the LTR-HSC selected using the Ho/

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. 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. Both cell fractions were previously characterized in vivo and in vitro. 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.
Rh method. Also, the number of descendant 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 cells can be inferred from studies of Wendling et al., 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. 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. 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 over unit time. Previous studies by our group and others have also shown that Tpo can act in synergy with Epo to affect erythropoiesis.17-19 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.

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