The recently cloned c-mpl ligand, thrombopoietin (Tpo), has been extensively characterized with regard to its ability to stimulate the growth, development, and plodity of megakaryocyte progenitor cells and platelet production in vitro and in vivo. Primitive hematopoietic progenitors have been shown to express c-mpl, the receptor for Tpo. In the present study, we show that Tpo efficiently promotes the viability of a subpopulation of Lin−Sca−1+ bone marrow progenitor cells. The ability of Tpo to maintain viable Lin−Sca−1+ progenitors was comparable to that of granulocyte colony-stimulating factor and interleukin-1, whereas stem cell factor (SCF) promoted the viability of a higher number of Lin−Sca−1+ progenitor cells when incubated for 40 hours. However, after prolonged (>40 hours) preincubation, the viability-promoting effect of Tpo was similar to that of SCF. An increased number of progenitors surviving in response to Tpo had megakaryocyte potential (37%), although almost all of the progenitors produced other myeloid cell lineages as well, suggesting that Tpo acts to promote the viability of multipotent progenitors. The ability of Tpo to promote viability of Lin−Sca−1+ progenitor cells was observed when cells were plated at a concentration of 1 cell per well in fetal calf serum-supplemented and serum-depleted medium. Finally, the DNA strand breakage elongation assay showed that Tpo inhibits apoptosis of Lin−Sca−1+ bone marrow cells. Thus, Tpo has a potent ability to promote the viability and suppress apoptosis of primitive multipotent progenitor cells.

© 1996 by The American Society of Hematology.

ALTHOUGH HEMATOPOIESIS involves a high turnover of mature blood cells, most of the earliest hematopoietic stem cells are thought to be quiescent in normal steady state marrow and only infrequently divide. Stimulatory hematopoietic growth factors, such as the colony-stimulating factors (CSFs), multiple interleukins (ILs), erythropoietin (Epo), stem cell factor (SCF), and flt3 ligand (FL), have been shown to stimulate the in vitro growth and differentiation of hematopoietic progenitor cells. The optimal clonogenic growth of primitive hematopoietic progenitors in general requires simultaneous activation through multiple cytokine receptors, whereas some cytokines have been shown to promote the viability of the same progenitors when acting individually. Specifically, previous studies have shown that hematopoietic growth factors, such as IL-3, SCF, granulocyte colony-stimulating factor (G-CSF), and IL-1, can support the survival of primitive hematopoietic progenitors and long-term repopulating cells in the absence of detectable colony formation. Furthermore, withdrawal of CSFs from CSF-dependent hematopoietic progenitor cell lines has been shown to induce programmed cell death or apoptosis.

c-mpl shows homology with other cytokine receptors and is a member of the hematopoietic cytokine receptor family. c-mpl knock-out mice have an 85% decrease in their number of platelets and megakaryocytes, but normal amounts of other hematopoietic cell types, suggesting that c-mpl (and its ligand) is a critical and selective regulator of megakaryocyte and platelet formation in vivo. The recently cloned c-mpl ligand, thrombopoietin (Tpo), has been shown to be involved in megakaryocytopoiesis and thrombopoiesis.

Tpo shows, in particular, homology with Epo, its ability to promote the viability of hematopoietic progenitor cells and, if so, to what degree surviving progenitors were committed to the megakaryocyte lineage or still multipotent. Because of the homology between Tpo and Epo and their corresponding receptors, we directly compared the ability of Tpo and Epo to act as viability factors on this progenitor cell population.

In the present report, we show that Tpo, but not Epo, as a single growth factor can promote the survival of a subtraction of the Lin−Sca−1+ progenitor cells and reduces the percentage of cells undergoing apoptosis in culture.

MATERIALS AND METHODS

Hematopoietic growth factors. Recombinant murine (rm) Tpo was generated as previously described. Recombinant human (rh) Tpo was generated as previously described. Tpo was generated as previously described.

From the Hipple Cancer Research Center, Dayton, OH; and ZytoGenetics Corp, Seattle, WA.

Submitted December 29, 1995; accepted May 29, 1996.

Address reprint requests to Ole J. Borge, MSc, Blood Cell Growth Factors Laboratory, Hipple Cancer Research Center, 4100 S Kettering Blvd, Dayton, OH 45439-2092.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.
Epo was kindly supplied by Boehringer Mannheim (Mannheim, Germany) and found to potently stimulate burst-forming unit-erythroid (BFU-E) colony formation in combination with SCF from Lin - BM cells (63 ± 4 BFU-E from 5,000 Lin - BM cells; mean of 3 experiments ± SEM). rhG-CSF and recombinant rat (rr) SCF were kindly provided by Dr Ian K. McNiece (Amgen Corp, Thousand Oaks, CA), rhFl, rhFl-7, and rhFl-1a were kindly supplied by Immunex (Seattle, WA) and rmFL-3 was from PeproTech Inc (Rocky Hill, NJ). Unless otherwise indicated, all growth factors were used at the following predetermined optimal concentrations, as determined by their ability to stimulate the growth of individually plated Lin - Sca-1 progenitor cells in combination with one other cytokine (IL-3 or SCF), except rrEpo, which was tested for its ability to stimulate BFU-E in combination with SCF from Lin - cells: rmTpo, 1,000 U/mL; rrEpo, 5 U/mL; rhG-CSF, 50 ng/mL; rrSCF, 100 ng/mL; rhFl, 50 ng/mL; rhFl-7, 100 ng/mL; rhFl-1a, 20 ng/mL; and rmFL-3, 20 ng/mL. All cytokines were used at the same concentration in multifactor combinations as well.

Enrichment and purification of Lin - Sca-1 BM cells. Lineage-depleted (Lin - ) BM cells were isolated from normal female C57BL mice (6 to 10 weeks old) according to previously described protocols. Briefly, femurs and tibias were gently crushed in a mortar. Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Walkersville, MD) supplemented with 5% fetal calf serum (FCS; BioWhittaker) was used as medium during the isolation. The cell suspension was filtered through a 70-μm mesh filter (Falcon; Becton Dickinson, Linkon Park, NJ). White blood cells were counted in a hemacytometer (red blood cells lysed with 10% Zap-oglobin II; Coulter Diagnostics, Hialeah, FL) and concentrated to 400 × 10^6 cells/mL. The cells were incubated at 4°C for 20 minutes in a cocktail of predetermined optimal concentrations of antibodies (the final concentration of each antibody was 2.5 μg/mL; RA-3682 (B220), RB6-8C5 (Gr-1), MAC-1, Lyt-2 (CD8), LY-1 (CD5), and L3T4 (CD4; all from Pharmingen, San Diego, CA); and Ter-119 (a kind gift from McNiece) supplemented with 5% FCS, concentrated to 400 × 10^6 cells/mL) or irrelevant isotype-matched control antibodies (a mixture of puriﬁed rat IgG2b and IgG3 [both Pharmingen] at the same [total] concentrations as the lineage-speciﬁc antibodies). After washing, cells were stained with a secondary goat antirat PE-conjugated antibody was added and incubated at 4°C for 20 minutes. Finally, the cells were washed and resuspended in a PI staining solution (see below). Direct viability assay) to discriminate between dead and viable cells. The cells were analyzed on a flow cytometer (FACSort; Becton Dickinson, San Jose, CA). Freshly isolated unfractonated BM cells were in two experiments included as a Lin - control. In both experiments, 90% to 95% of the unfractonated BM cells were Lin - .

Single-cell proliferation assay. Lin - Sca-1 cells were seeded in Terasaki plates (Nunc, Kamstrup, Denmark) at a concentration of 1 cell per well in 20 μL of IMDM supplemented with 20% FCS (complete IMDM) or serum-depleted medium (X-vivo 15; BioWhittaker) supplemented with 1% detoxified bovine serum albumin (BSA; StemCell Technologies Inc, Vancouver, British Columbia, Canada). Both media contained 100 U/mL penicillin (BioWhittaker), 100 U/mL streptomycin (BioWhittaker), and 3 mg/mL L-glutamine (BioWhittaker). Wells were scored for cell growth (>3 cells) after 10 to 12 days of incubation at 37°C and 5% CO₂ in air. In some experiments, individual colonies covering more than 10% of the well were sampled, transferred to slides in a cytospin centrifuge (Shandon, Cheshire, UK), and examined morphologically after Giemsa (Sigma, St Louis, MO) staining.

Identification of megakaryocytes: Acetylcholinesterase (AchE) staining. AchE is a marker enzyme of murine megakaryocytes. AchE activity was detected by a modification of a previously described method. Briefly, Lin - Sca-1 cells were cultured in serum-depleted medium with different cytokine combinations. After 11 days of incubation, the cells were stained directly in almost completely dried wells. Cells were incubated with acetylcholine substrate (all chemicals from Sigma) overnight at 37°C and 5% CO₂ in air. Wells that contained more than three megakaryocytes, based on color change, were scored as positive. The megakaryocyte content was also confirmed by parallel experiments in which individual colonies were transferred to slides in a cytospin centrifuge, fixed, and stained with Giemsa.

Replating of primary colonies with a selective megakaryocyte stimulus. Lin - Sca-1 cells were cultured at a density of 1 cell per well in 10 μL serum-depleted medium supplemented with Tpo, SCF, or a strong proliferative cocktail (Tpo + SCF + G-CSF + IL-3). After 40 hours of preincubation, 10 μL of serum-depleted medium containing cocktail was added to each well to yield predetermined optimal concentrations. The cells were incubated for an additional 6 days at 37°C and 5% CO₂ in air. In each experiment, and for each
TPO PROMOTES VIABILITY OF PROGENITOR CELLS

group, 24 colonies covering more than 10% of the well were picked, washed, and replated individually in predetermined optimal concentrations of Tpo + IL-3 in 96-well plates. The wells were examined for megakaryocyte content by AChE staining after an additional 8 days of incubation at 37°C and 5% CO₂ in air.

Evaluation of B-cell potential of Lin-Sca-1⁺ progenitors surviving in response to Tpo. We have recently shown that FL + IL-7 potentially and selectively promote pro-B–cell development from uncommitted Lin-Sca-1⁺ progenitor cells, an effect that can be further enhanced by SCF. To evaluate the B-cell potential of Lin-Sca-1⁺ cells (plated individually as described above) surviving in response to Tpo for 40 hours, we therefore added predetermined optimal concentrations of SCF + FL + IL-7. After a total of 10 days of incubation, wells containing colonies covering at least 10% of the well were replated in fresh medium supplemented with SCF + FL + IL-7 in round-bottomed 96-well plates to optimize B-cell development. After an additional 20 days of incubation, wells containing sufficient cell numbers were analyzed for B220 expression. Colonies were picked and washed, and purified rat IgG (30 µg/mL) was added to a final dilution factor of 1:20, and 50 µL/well was counted by Trypan blue staining and resuspended in 10 µg/mL PI (Sigma), 100 µM RNAse A (Sigma), and 1% FCS in phosphate-buffered saline (PBS; BioWhittaker). The cells were incubated for 15 minutes at 4°C. The cells were washed, resuspended in PI (to exclude dead cells; see below, Direct viability assay), and analyzed on a flow cytometer (FACSort).

Single-cell indirect viability assay: Delayed addition studies. Lin-Sca-1⁺ cells were seeded as previously described in Terasaki plates at a concentration of 1 cell per well in a volume of 10 µL complete IMDM or serum-depleted medium with putative viability factors at predetermined optimal concentrations. The cells were pre-incubated for different periods of time at 37°C and 5% CO₂ in air before an additional 10 µL of medium containing a potent proliferative stimulus was added to each well. Wells were scored for cell growth (>3 cells) after a total of 10 to 12 days of incubation at 37°C and 5% CO₂ in air. The culture scheme is shown in Fig 1.

Direct viability assays. To directly assess the effect of Tpo on viability, 10,000 Lin-Sca-1⁺ cells were incubated in serum-depleted medium alone or supplemented with Tpo. After 40 hours of incubation, viable cells were counted by Trypan blue staining and resuspended in 10 µg/mL PI (Sigma), 100 µM RNAse A (Sigma), and 1% FCS in phosphate-buffered saline (PBS; BioWhittaker). The cells were incubated for 5 minutes at room temperature to expose the plasma membrane before being analyzed on a flow cytometer (FACSort).

Apoptosis assay. Apoptotic cells were detected using a modification of a previously described method. Briefly, 50,000 Lin-Sca-1⁺ cells were incubated in serum-depleted medium alone or supplemented with Tpo for 40 hours. The cells were pelleted in a microcentrifuge and fixed in 1% methanol-free formaldehyde (Poly-Science Inc, Warrington, PA) for 15 minutes on ice, pelleted, and resuspended in 70% EtOH and stored at −20°C until used. The cells were washed once in PBS (PBS containing 1% FCS). A TdT kit (In Situ Death Detection Kit; fluorescein) from Boehringer Mannheim was used. After washing, the cells were resuspended in 45 µL label solution (containing fluorescein-dUTP and optimized buffer concentrations). Ten microliters was removed as a negative control and to the remaining 35 µL, 4 µL of an enzyme solution (containing terminal deoxynucleotidyl transferase (TdT)) was added. Both samples were incubated for 60 minutes at 37°C. Finally, 300 µL PBSS was added, and the cells were resuspended and analyzed on a flow cytometer (FACSort).

Statistical analysis. Student’s t-test was used for statistical analysis.

RESULTS

The ability of Tpo to promote viability of Lin-Sca-1⁺ BM progenitor cells in FCS-containing and serum-depleted cultures as determined by an indirect assay: Comparison with other putative viability factors. IL-1, SCF, G-CSF, and Epo have viability-promoting effects on different classes of hematopoietic progenitors. The ability of Tpo to potentially promote viability of Lin-Sca-1⁺ cells was compared with these cytokines in both FCS-containing (Fig 2) and serum-depleted media (Table 1). To avoid indirect effects, cells were seeded at a density of 1 cell per well in Terasaki plates. After 40 hours of preincubation in complete IMDM alone, almost no cells were able to initiate clonal growth after the addition of a strong proliferative cocktail (ie, Tpo, SCF, G-CSF, and IL-3). In contrast, if this cocktail was added at the beginning of culture, 78 clones were formed from 300 Lin-Sca-1⁺ cells. SCF, which is a strong viability factor for primitive progenitors, promoted the survival of 59% of the progenitors responsive to the cocktail. Also, in agreement with previous studies, both IL-1 and G-CSF promoted the viability of Lin-Sca-1⁺ progenitors despite having little or no growth promoting activity when added alone. Like IL-1 and G-CSF, Tpo alone had little proliferative activity and stimulated the formation of only 1 cluster (<50 cells) and no colonies, but promoted the survival of 10 Lin-Sca-1⁺ progenitors after 40 hours of preincubation. Thus, Tpo promoted the survival of 13% of the cocktail-responsive progenitors in FCS-containing medium and to a similar degree as did G-CSF and IL-1. In contrast, Epo, which is known to promote the viability of committed erythroid progenitor cells, had no viability-promoting effect on Lin-Sca-1⁺ progenitor cells in complete IMDM.

In contrast to the results observed in FCS-supplemented medium, Tpo alone promoted the formation of 14 clusters in serum-depleted medium (Table 1), and 37% of the cocktail-responsive Lin-Sca-1⁺ progenitors remained responsive after 40 hours of preincubation in Tpo alone, suggesting that Tpo had a viability-promoting activity on a subset of progenitors that did not grow in response to Tpo alone. As in FCS-supplemented cultures, Epo had, unlike Tpo, no growth-stimulating or viability-promoting activity, whereas 70% of the cocktail-responsive progenitors could be recovered after 40 hours of preincubation in SCF alone. The size distribution of the clones formed in response to Tpo + SCF + G-CSF + IL-3 from Lin-Sca-1⁺ progenitors surviving in response to Tpo, IL-1, G-CSF, or SCF was similar to that formed from freshly isolated Lin-Sca-1⁺ progenitors. Thus, Tpo, but not Epo, promotes the viability of a subfraction of Lin-Sca-1⁺ progenitor cells. The Tpo-stimulated survival of Lin-Sca-1⁺ cells occurred in a concentration-dependent manner in serum-depleted medium (Fig 3). A weak viability signal was seen at 12 U/mL, whereas 333 to 1,000 U/mL resulted in an optimal effect. Similar results were observed in serum-supplemented cultures (data not shown).

The kinetics of Tpo-promoted viability of Lin-Sca-1⁺ pro-
To determine whether the viability-promoting effect of Tpo was prolonged, Lin'Sca-1' cells were preincubated in Tpo, SCF, or serum-depleted medium alone and, after different periods of time, a cocktail (ie, Tpo + SCF + G-CSF + IL-3) was added. The number of progenitors responsive to this cocktail was predominantly reduced during the first 24 hours in all three groups (Fig 4). Whereas SCF was a significantly stronger viability factor than Tpo at 24 and 40 hours ($P < .01$ and $P < .05$, respectively), no significant difference could be observed at 72 hours ($P = .17$). Of particular interest was the observation that there was no significant reduction in viable/responsive progenitors after 56 hours ($P = .12$) and 72 hours ($P = .24$) of preincubation with Tpo when compared with 40 hours of preincubation.

To ensure that the viability-promoting effect of Tpo was observed on single cells at the initiation of culture and that these had not multiplied after 40 hours of incubation, wells containing 1 single cell were identified 2 hours after plating (to allow cells to sediment) and after 40 hours of preincubation in Tpo alone. Only 9% ± 2% of the wells initially containing 1 cell had proliferated (>1 cell) after 40 hours of incubation. In five experiments, a mean of $16% ± 2%$ of the wells identified to contain a single cell directly after plating as well as containing 1 (or no) cell after 40 hours of preincubation produced clones after an additional 10 days of incubation in Tpo + SCF + G-CSF + IL-3. Other experiments showed that Lin'Sca-1' cells remained negative for lineage-specific cell surface antigens and positive for Sca-1 after 40 hours of incubation in Tpo (Fig 5).

**Megakaryocyte potential of Lin'Sca-1' progenitor cells surviving in response to Tpo.** It has been suggested that Tpo might act selectively on the megakaryocyte lineage; thus, it might selectively enhance survival of progenitors with a megakaryocyte potential. The absolute number of megakaryocyte-containing clones formed in response to the strong proliferative cocktail (ie, Tpo + SCF + G-CSF + IL-3) was slightly reduced in cultures preincubated in Tpo alone for 40 hours as compared with cultures stimulated with the cocktail at the initiation of culture (14 ± 2 and 17 ± 1, respectively; mean ± SEM of 6 experiments). However,
TPO PROMOTES VIABILITY OF PROGENITOR CELLS

The ability of Tpo and other cytokines to promote viability of Lin 'Sca-1' progenitor cells in FCS-containing medium. Lin 'Sca-1' cells were cultured at a density of 1 cell per well in 10 μL complete IMDM and predetermined optimal concentrations of the indicated cytokines. After 40 hours of preincubation, 10 μL complete IMDM medium containing Tpo + SCF + G-CSF + IL-3 to yield predetermined optimal concentrations was added to each well. Clones (>3 cells) were scored after a total of 10 to 12 days of incubation at 37°C and 5% CO2 in air. The results represent the means ± SEM of the total number of clones per 300 Lin 'Sca-1' cells of five individual experiments. Three hundred wells were scored in each experiment. When 300 Lin 'Sca-1' cells were cultured for 10 to 12 days in the absence of cytokines or in the presence of Epo or IL-1, no clones were produced, whereas Tpo, G-CSF, and SCF individually resulted in the growth of 1 ± 1, 2 ± 2, and 12 ± 2 clones, respectively. The cocktail containing Tpo + SCF + G-CSF + IL-3 stimulated the formation of 78 ± 5 clones.

Interestingly, Tpo appeared to selectively promote the survival of Lin 'Sca-1' progenitor cells with a megakaryocyte potential, because 38% of the surviving progenitors after preincubation with Tpo formed megakaryocyte-containing clones, as compared with 23% of the progenitors responsive to the cocktail at the initiation of culture (P < .01; Fig 6). The ability of Tpo to selectively promote survival of progenitors capable of producing megakaryocytes was significantly stronger than SCF (P < .05). In fact, the megakaryocyte potential of Lin 'Sca-1' progenitor cells surviving in response to SCF did not significantly differ from freshly isolated Lin 'Sca-1' cells (P = .15).

Next, the content of other myeloid cell lineages in colonies formed by Lin 'Sca-1' cells surviving for 40 hours in Tpo was investigated (Fig 7). These studies showed the presence of colonies containing exclusively macrophages and/or granulocytes (39%), undifferentiated blast cell colonies (19%), pure megakaryocyte colonies (1%), mixed megakaryocyte/blast cell colonies (15%), and mixed megakaryocyte/granulocyte/macrophage colonies (27%). Thus, although these studies showed that Tpo preferentially promoted the survival of progenitors with a megakaryocyte potential, they also showed that a high percentage (57%) of the progenitors surviving in Tpo did not produce megakaryocytes in response to the cocktail (ie, Tpo + SCF + G-CSF + IL-3). It has been shown that most cytokines capable of stimulating the growth of Lin 'Sca-1' progenitor cells in vitro preferentially promote their granulocyte/macrophage differentiation and that their potential for forming cells of other lineages such as B cells frequently can be detected only after replating in more lineage-selective cytokines. Thus, we argued that replating of clones with a more megakaryocyte-specific cytokine combination (Tpo + IL-3) might better uncover the megakaryocyte potential of Lin 'Sca-1' progenitors surviving in response to Tpo. Accordingly (in 5 separate experiments), after 40 hours of preincubation in Tpo alone and subsequently 5 days of incubation in a cocktail (ie, Tpo + SCF + G-CSF + IL-3), clones (covering more than 10% of the well) were washed, replated in Tpo + IL-3, and incubated for an additional 8 days before the megakaryocyte content was examined using AChE staining. A total of 52% ± 8% of the progenitors that originally survived in the presence of Tpo formed megakaryocyte-containing colonies in response to Tpo + IL-3, as compared with 38% ± 2% megakaryocyte-containing clones formed when the secondary incubation in

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Degree of Proliferation*</th>
<th>Total Clones/300 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preincubation After</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1          2      3   4</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>0          0      0    0</td>
</tr>
<tr>
<td>None</td>
<td>Cocktail</td>
<td>1          1      1     1</td>
</tr>
<tr>
<td>Cocktail</td>
<td>8          8     26    53 96</td>
<td>(3)</td>
</tr>
<tr>
<td>Epo</td>
<td>Epo</td>
<td>0          0      0    0</td>
</tr>
<tr>
<td>Epo</td>
<td>Cocktail</td>
<td>1          1      0     3</td>
</tr>
<tr>
<td>Tpo</td>
<td>Tpo</td>
<td>12         2      0    14</td>
</tr>
<tr>
<td>Tpo</td>
<td>Cocktail</td>
<td>4          4      7     18</td>
</tr>
<tr>
<td>G-CSF</td>
<td>G-CSF</td>
<td>1          0      0     1</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Cocktail</td>
<td>1          2      5     12</td>
</tr>
<tr>
<td>IL-1</td>
<td>IL-1</td>
<td>0          0      0    0</td>
</tr>
<tr>
<td>IL-1</td>
<td>Cocktail</td>
<td>6          1      8     11</td>
</tr>
<tr>
<td>SCF</td>
<td>SCF</td>
<td>22         10     4     0</td>
</tr>
<tr>
<td>SCF</td>
<td>Cocktail</td>
<td>5          5      20    31</td>
</tr>
</tbody>
</table>

Lin 'Sca-1' cells were cultured at a density of 1 cell per well in 10 μL serum-depleted medium (Materials and Methods) and the indicated cytokines. After 40 hours of preincubation, 10 μL serum-depleted medium and the indicated cytokines to yield predetermined optimal concentrations were added to each well. Clones were scored based on size according to the scoring criteria described below, after a total of 10 to 12 days of incubation at 37°C and 5% CO2 in air. The results represent the means for each size category and means (SEM) for total number of clones per 300 Lin 'Sca-1' cells of six individual experiments, with 300 wells scored in each experiment. Cocktail contains Tpo + SCF + G-CSF + IL-3.

* Scoring criteria: 1, wells with 3 to 50 cells; 2, colonies with more than 50 cells, but covering less than 10% of well; 3, colonies covering 10% to 50% of well; 4, more than 50% of the well covered by cells.

* P < .01 when compared with putative viability factor alone for full incubation period.
Tpo + IL-3 was not performed. However, this difference did not reach statistical significance (P = .1).

The reason why only 37% of the cocktail-responsive Lin"Sca-1" progenitor cells survived in response to Tpo might simply be due to the lack of c-mpl expression on some of these progenitors. Thus, Lin"Sca-1" progenitors surviving in Tpo were next rescued by SCF + Tpo or IL-3 + Tpo to more selectively target c-mpl expressing Lin"Sca-1" cells (Table 2). Interestingly, although the cocktail stimulated the growth of a higher number of freshly isolated Lin"Sca-1" progenitor cells than either IL-3 + Tpo or SCF + Tpo (101, 65, and 64, respectively), the growth of cells preincubated for 40 hours in Tpo alone did not differ in response to these three cytokine combinations. Accordingly, 57% of SCF + Tpo-responsive and 54% of IL-3 + Tpo-responsive Lin"Sca-1" progenitor cells survived for 40 hours in Tpo alone, as compared with 37% of the cocktail-responsive progenitors (Table 1).

The results described above established that the Lin"Sca-1" progenitors surviving in response to Tpo had a potential to differentiate into different myeloid cell lineages. We next investigated whether some might also have a potential to develop towards the B-cell lineage. For this purpose, we used a recent observation showing that FL + IL-7 in the absence or presence of SCF selectively promote development of long-term pro-B–cell cultures from Lin"Sca-1" progenitor cells. In a total of three experiments after 40 hours of preincubation in Tpo, 65 ± 2 cells (of 600 Lin"Sca-1" cells plated individually) survived in response to Tpo and formed clones in response to the subsequent addition of SCF + FL + IL-7. Of these, 40 ± 1 clones covered more than 10% of the well after 10 days of incubation, at which time they were replated in round-bottomed 96-well plates to optimize B-cell development. After an additional 20 days of incubation in SCF + FL + IL-7, 9 ± 2 wells containing viable cells were analyzed by flow cytometry for B220 expression. Of these, 8 ± 1 clones expressed high levels of B220, whereas 1 ± 1 was B220−. Thus, Tpo promotes the survival of Lin"Sca-1" progenitor cells capable of long-term production of B220− cells.

All Lin"Sca-1" progenitors surviving in response to Tpo are c-kit+. Previous studies have suggested that all long-term reconstituting and clonogenic Lin"Sca-1" progenitor cells are c-kit+. In agreement with this, 72 ± 8 of 180 Lin"Sca-1"c-kit+ cells formed clones in response to Tpo + SCF + G-CSF + IL-3, whereas only 1 ± 1 of 180 Lin"Sca-1"c-kit− cells was clonogenic when stimulated with the same cytokine combination (P < .05). Accordingly, a significantly
TPO PROMOTES VIABILITY OF PROGENITOR CELLS

1 Sca-1.

Log fluorescence intensity

Fig 5. Expression of lineage-specific cell surface antigens and Sca-1 on Lin`Sca-1' cells incubated in the presence of Tpo for 40 hours.

In three individual experiments, 80,000 Lin`Sca-1' cells were incubated in serum-depleted medium supplemented with Tpo for 40 hours. Cells were harvested and phenotyped as described (Materials and Methods). (A) Sca-1 expression. (B) Lineage expression. The open histograms in (A) and (B) represent irrelevant control antibody staining.

Fig 6. Megakaryocyte production of Lin`Sca-1' progenitor cells preincubated in Tpo or SCF. Lin`Sca-1' cells were cultured at a density of 1 cell per well in 10 μL serum-depleted medium and optimal concentrations of the indicated cytokines. After 40 hours of preincubation in Tpo, 10 μL of serum-depleted medium containing Tpo + SCF + G-CSF + IL-3 (cocktail) was added to each well to yield predetermined optimal concentrations. After a total of 10 days of incubation at 37°C and 5% CO₂ in air, 20 colonies (covering more than 10% of the well) were picked and transferred to slides in a cytopsin centrifuge, fixed, and Giemsa-stained. Colonies containing more than 50% blasts (Bl) were scored as a Bl colony. Colonies containing more than 50% of either granulocytes (G), macrophages (M), or megakaryocytes (Mk) were classified as G, M, and Mk colonies, respectively. Colonies containing a mixture of G and M were scored as GM colonies. A Bl colony containing 1 or more Mk were classified as a Bl + Mk colony. A mixture of Mk, G, and/or M was classified as a Mk-mix colony. Results represent the mean percentages ± SEM for distribution of different colony types in four separate experiments. For each group, 20 colonies were examined in each experiment.

(P < .05) increased frequency of progenitors surviving in response to Tpo was observed in Lin`Sca-1' c-kit' BM cells (17%; Fig 8) as compared with unfractionated Lin`Sca-1' cells (11%; Table I).

Direct viability assays. Viability of Lin`Sca-1' cells were next evaluated by Trypan blue and PI exclusion (Materials and Methods) after 40 hours of incubation of Lin`Sca-1' cells in serum-depleted medium alone or supplemented with Tpo. As determined by Trypan blue exclusion, 5,200 ± 700 of the 10,000 cells plated originally in the presence of Tpo remained viable after 40 hours of incubation, as compared with 4,500 ± 800 in medium alone (Fig 9A; P = .16). PI analysis, which is considered a more sensitive viability assay than Trypan blue exclusion, showed that 38% ± 3% of the remaining cells in Tpo-supplemented cultures were viable (no PI-uptake), as compared with only 21% ± 1% in cultures containing serum-depleted medium alone (P < .05; Fig 9B).

Tpo inhibits apoptosis of Lin`Sca-1' BM cells. Epo and other cytokines increase the viability of hematopoietic progenitor cells by preventing apoptosis.14,26 To examine whether the increased viability of Lin`Sca-1' BM cells ob-
than 50 cells, but covering less than 10% of well; 3, colonies covering
contains Tpo + SCF + G-CSF + IL-3.
cells were incubated for 40 hours in serum-depleted medium
dUTP in DNA strand breaks (Fig 10). The percentage of
of apoptosis using TdT for the incorporation of fluorescein-
centrations were added to each well. Clones were scored based on
of cytokines. After 40 hours of preincubation, 10 pL serum-depleted me-
density of 1 cell per well in 10 μL
serum-depleted medium (Materials and Methods) and the indicated
concentrations were added to each well. Cells were scored based on
size according to the scoring criteria described below, after a total of
10 to 12 days of incubation at 37°C and 5% CO2 in air. The results
represent the means for each size category and means (SEM) for
total number of clones per 300 Lin ‘Sca-1’ cells of three individual
experiments, with 300 wells scored in each experiment. Cocktail con-
tains Tpo + SCF + G-CSF + IL-3.

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Degree of Proliferation</th>
<th>Total Clones/300 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation</td>
<td>After Preincubation</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>Cocktail</td>
<td>2</td>
</tr>
<tr>
<td>Tpo</td>
<td>Cocktail</td>
<td>4</td>
</tr>
<tr>
<td>Tpo</td>
<td>Tpo, SCF</td>
<td>12</td>
</tr>
<tr>
<td>Tpo, SCF</td>
<td>Tpo, SCF</td>
<td>23</td>
</tr>
<tr>
<td>Tpo, IL-3</td>
<td>Tpo, IL-3</td>
<td>12</td>
</tr>
<tr>
<td>Tpo, IL-3</td>
<td>Tpo, IL-3</td>
<td>17</td>
</tr>
</tbody>
</table>

Lin ‘Sca-1’ cells were cultured at a density of 1 cell per well in 10 μL
serum-depleted medium in the absence or presence of Tpo and examined for signs of
apoptosis using TdT for the incorporation of fluorescein-
dUTP in DNA strand breaks (Fig 10). The percentage of
apoptotic cells (incorporated fluorescein-dUTP) was signifi-
cantly reduced (P < .005) when cells were incubated in Tpo
as compared with when the cells were incubated in medium
alone. The relatively high percentage of viable Lin ‘Sca-1’
cells observed here can probably be explained by previous
studies showing that cells undergoing apoptosis in culture
subsequently undergo secondary necrosis and disintegrate
rapidly after apoptosis.44,45 This is in agreement with our
data indicating that only approximately 50% of the initial
cell population was recovered after 40 hours of incubation
in the absence or presence of Tpo. In conclusion, Tpo inhibits
apoptosis of Lin ‘Sca-1’ BM cells in culture.

**DISCUSSION**

Recently Tpo, the ligand for c-mpl was cloned, and multi-
ple studies have already shown its ability, alone and in
combination with other cytokines, to potently promote the in
vitro growth and differentiation of megakaryocyte progeni-
tors and its ability to act in a lineage-specific manner to
potently stimulate platelet production in vivo.19,24,27,20
Whereas it is clear that c-mpl and its ligand play a critical
role in promoting megakaryocyte and platelet production, it
has not yet been established whether Tpo might act to pro-
mote the viability of hematopoietic progenitor cells.

We addressed whether Tpo could promote the viability of
murine Lin ‘Sca-1’ BM cells, which have been shown to be
highly enriched in primitive hematopoietic progenitor cells
important for short-term as well as long-term reconstitu-
tion.14,16,27 It has previously been shown that some cytokines,
particularly SCF, can potently promote the viability of primiti-
ve progenitors such as Lin ‘Sca-1’ cells in vitro while stimu-
lating limited proliferation.9,12,37,39

In the present study, we show that Tpo also has the ability
to promote the in vitro viability of Lin ‘Sca-1’ progenitor
cells in FCS-containing and serum-depleted medium when
plated at 1 cell per well, suggesting a direct effect on the
progenitors. The ability of Tpo alone to promote the viability
(and the growth) of Lin ‘Sca-1’ progenitor cells was more
pronounced in serum-depleted than in FCS-containing me-
dium. Specifically, after 40 hours of incubation in Tpo alone
in serum-depleted or FCS-supplemented medium, 37% and
13%, respectively, of the progenitors that could originally
respond to Tpo + SCF + G-CSF + IL-3 (cocktail) were
still responsive to the same cytokine combination, whereas
few or no progenitors survived in medium alone. Although
the reason for this difference between FCS-containing and
serum-depleted medium remains unclear, we have shown
that FCS contains small amounts of active transforming
growth factor-β that can negatively influence the growth
and the viability of primitive murine progenitor cells.37,46
Regardless, both the studies in FCS-supplemented and se-
rum-depleted medium show that Tpo, when acting alone,
can promote the viability of a subfraction of Lin ‘Sca-1’
progenitor cells that cannot initiate clonal growth in the
absence of other cytokines. Such a viability-promoting effect
of Tpo was specifically shown on cells that had not
multiplied after 40 hours and that maintained the Lin ‘Sca-
1’ phenotype. However, it cannot be excluded that the cells
might have differentiated somewhat, because it has been
shown that, although cells with the Lin ‘Sca-1’ phenotype
can be expanded (in vivo), they do appear to loose stem cell
activity.27

In addition to its viability-promoting effect, Tpo alone
can promote the clonal growth of a fraction of Lin ‘Sca-1’
progenitor cells in serum-depleted medium. These findings
suggest that a subfraction of Lin ‘Sca-1’ BM progenitor cells
express c-mpl, in agreement with a previous study showing
that 50% of AA4 ‘Sca-1’ murine fetal liver cells express c-
mpl and that suspension cultures of 10,000 such cells could
produce a low amount of megakaryocytes in response to Tpo
alone.30 In other studies in our laboratory, we have recently
shown that this limited growth response of Lin ‘Sca-1’ pro-
genitor cells to Tpo alone can be enhanced by other early
acting cytokines to promote multilineage clonal growth.29
Whether these c-mpl–expressing primitive progenitors in-
clude the long-term reconstituting stem cells remains to be
determined.

It is possible that not all Lin ‘Sca-1’ progenitors responsi-
tive to the potent growth factor combination containing Tpo
+ SCF + G-CSF + IL-3 express c-mpl and that, accordingly,
we might underscore the potential of Tpo to promote the
viability of c-mpl–expressing Lin ‘Sca-1’ progenitor cells.
Thus, we also investigated the ability of Tpo to promote the
viability of Lin ‘Sca-1’ progenitor cells responsive to SCF
+ Tpo and IL-3 + Tpo to more selectively recruit c-mpl-
expressing progenitors. Interestingly, Tpo supported the sur-
TPO PROMOTES VIABILITY OF PROGENITOR CELLS

Fig 8. Tpo and SCF as viability factors for Lin~Sca-1~c-kit~ cells. Lin~Sca-1~c-kit~ cells were cultured at a density of 1 cell per well in 10 μL serum-depleted medium and predetermined optimal concentrations of the indicated cytokines. After 40 hours of preincubation, 10 μL of serum-depleted medium containing Tpo + SCF + G-CSF + IL-3 to yield predetermined optimal concentrations was added to each well. Clones (>3 cells) were scored after a total of 10 to 12 days of incubation at 37°C and 5% CO2 in air. The results represent the means ± SEM for total number of clones per 180 Lin~Sca-1~c-kit~ cells of three individual experiments, with 180 wells scored in each experiment. Here, 11 ± 3 and 24 ± 0 clones were formed in response to Tpo and SCF alone, respectively. *Cocktail contains Tpo + SCF + G-CSF + IL-3.

Fig 9. Direct viability assays. Ten thousand Lin~Sca-1~ cells were incubated in serum-depleted medium for 40 hours in the presence or absence of Tpo. Viable cells were counted in a microscope using Trypan blue exclusion (A) before being resuspended in PI-staining solution and analyzed by flow cytometry (B), as described in the Materials and Methods. The results represent the mean ± SEM from four individual experiments.

vival of more than 50% of these progenitors, and, although the number of colonies formed by freshly isolated Lin~Sca-1~ cells in response to Tpo + SCF + G-CSF + IL-3 was higher than that of those that responded to either SCF + Tpo or IL-3 + Tpo, the number of progenitors responsive to each of the three combinations surviving in response to Tpo was similar.

Of the cytokines identified to date, SCF has been implicated as the most potent viability factor for primitive hematopoietic progenitor cells.10,12,27,39 The present study confirms that SCF promotes the viability of a large fraction of Lin~Sca-1~ progenitor cells. Interestingly, whereas the ability of SCF to promote the viability of Lin~Sca-1~ progenitor cells was significantly better than that of Tpo after 40 hours of incubation in medium alone, only a slight (nonsignificant) difference could be observed after more prolonged incubation (72 hours) with these two viability factors. In addition, the ability of Tpo to promote the viability of Lin~Sca-1~ progenitors (for 40 hours) was comparable to that of IL-1 and G-CSF, two other cytokines implicated as viability factors for progenitor cells.7,13 Thus, Tpo falls into a class of cytokines capable of promoting the viability of primitive hematopoietic progenitor cells.

Epo and its receptor have homology with Tpo and c-mpl, respectively, and Epo is the principal and selective regulator of erythropoiesis as Tpo is now believed to be for megakaryocytopoiesis.24,25 In addition, Epo is a potent viability factor for committed erythroid progenitor cells.49 Accordingly, it was of interest to address whether Tpo and Epo had similar effects on the viability of Lin~Sca-1~ progenitor cells. Interestingly, and in contrast to the potent viability-promoting activity of Tpo, Epo had no effect on the viability of Lin~Sca-1~ progenitor cells in FCS-supplemented or serum-depleted medium. This lack of effect of Epo on the viability and the growth48 of Lin~Sca-1~ progenitor cells could be due to the lack of Epo receptor expression on primitive hematopoietic progenitor cells. Although the Epo receptor expression on Lin~Sca-1~ BM cells has not been investi-
gated, it has been suggested that even primitive erythroid progenitor cells might not express the Epo receptor.\textsuperscript{39,50}

It was of particular interest to address to what degree the subfraction of Lin''Sca-1'' progenitor cells surviving in response to Tpo had megakaryocyte potential. Tpo appeared to selectively promote the survival of Lin''Sca-1'' progenitors with a megakaryocyte potential, because 38\% of the cocktail-responsive progenitors surviving in response to Tpo formed megakaryocyte-containing clones as compared with only 23\% of the freshly isolated Lin''Sca-1'' progenitors responsive to the same growth factor combination. However, 62\% of the progenitors surviving in response to Tpo did not produce megakaryocytes, and, although the number of megakaryocyte-producing progenitors surviving in response to Tpo was increased to 52\% when replated in a more megakaryocyte-selective cytokine combination (SCF + Tpo or IL-3 + Tpo), a high number of surviving progenitors produced cells of other myeloid lineages as well, some without the presence of megakaryocytes. This was confirmed both by AchE staining and morphologic evaluation of Giemsa-stained cytosin preparations. Although this could be due in part to the strict in vitro requirements for optimal megakaryocyte growth and development, it does at least reflect the multipotentiality of the Lin''Sca-1'' progenitor cells surviving in response to Tpo and that most of these Tpo-responsive progenitors are capable of producing other cells as well. In fact, only 1\% of the Lin''Sca-1'' progenitors surviving in response to Tpo produced exclusively megakaryocytes in response to Tpo + SCF + G-CSF + IL-3. Based on recent studies in our laboratory,\textsuperscript{56} we also evaluated whether a subpopulation of Lin''Sca-1'' progenitor cells surviving in response to Tpo might also have B-cell potential. Interestingly, a fraction of Lin''Sca-1'' cells surviving in response to Tpo for 40 hours produced high numbers of B220\'' cells after prolonged incubation in SCF + FL + IL-7. Thus, although the effects of Tpo administration to normal mice in vivo as well as studies in c-mpl-deficient mice clearly show the selectivity of Tpo for the megakaryocyte cell lineage, the present studies suggest that Tpo in combination with other cytokines might also be capable of stimulating the production of other cell lineages from multipotent progenitor cells.

A potential clinical significance of these findings is supported by recent studies showing that Tpo treatment of myeloablated mice (ie, depleted of more committed progenitors), in addition to promoting platelet recovery, also results in the improved recovery of red blood cells and other white cell lineages.\textsuperscript{51,55} It also seems likely that Tpo might prove useful in the ex vivo expansion of stem cells. Furthermore, a physi-
ologic relevance of the present study is supported by a recent study of progenitor cells in c-mpl-deficient mice showing a marked reduction in the number of progenitors of multiple hematopoietic lineages, leading the investigators to conclude that Tpo might act on the pluripotent stem cell.22

It is widely accepted that normal hematopoiesis involves apoptosis and its regulation and that primitive hematopoietic progenitors can undergo apoptosis.23,26,44,45 Many cytokines prevent the apoptotic cell death of primitive progenitor cells,24,26,44,45 and we show here for the first time that Tpo can enhance viability and inhibit the apoptotic cell death of Lin-Sca-1+ BM cells in vitro. However, because cell heterogeneity remains a limitation and challenge in studies using populations of enriched normal primitive hematopoietic progenitor cells,25,26,44,45 the indirect viability assay (ie, delayed addition studies) described here and by others3,9,12,37,39 will remain an essential assay reflecting the viability of primitive hematopoietic progenitor cells,25,26,44,45 do not necessarily reflect clonogenic-functional progenitors. In addition, as shown elsewhere,44,45 the present findings are consistent with apoptotic cells rapidly undergoing secondary necrosis and disintegrating in culture. Thus, the extent of apoptosis in the cultures of Lin-Sca-1+ cells is probably underestimated.

In conclusion, we have shown that Tpo potently promotes the viability of a subpopulation of Lin-Sca-1+ progenitor cells with a potential to produce megakaryocytes and other cell lineages, showing that Tpo, in addition to its ability to selectively promote the growth and differentiation of committed megakaryocyte progenitor cells, has a viability-promoting effect on progenitors that have not yet committed to the megakaryocyte cell lineage.

ACKNOWLEDGMENT

We thank Heide Kammer and O. Joseph Trask, Jr for help with isolation and sorting of BM cells, Emilio Barbera-Guillem and Jose Cardier for their expert advice in establishing the acetylcholinesterase staining assay, Zbigniew Darzynkiewicz for helpful suggestions regarding apoptosis assays, and the staff at Hipple Office Support Center and AlphaMed Press for preparing this manuscript.

REFERENCES


36. Veiby OP, Lyman SD, Jacobsen SEW: Combined signaling through IL-7 receptor and flt3 but not c-kit potently and selectively promotes B cell commitment and differentiation from uncommitted murine bone marrow progenitor cells. Blood (in press)


42. Ikuta K, Weissman IL: Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc Natl Acad Sci USA 89:1502, 1992


44. Lotem J, Sachs L: Hematopoietic cytokines inhibit apoptosis induced by transforming growth factor beta 1 and cancer chemotherapy compounds in myeloid leukemic cells. Blood 80:1750, 1992


From www.bloodjournal.org by guest on November 11, 2017. For personal use only.
Thrombopoietin, but not erythropoietin promotes viability and inhibits apoptosis of multipotent murine hematopoietic progenitor cells in vitro

OJ Borge, V Ramsfjell, OP Veiby, MJ Jr Murphy, S Lok and SE Jacobsen