Thrombopoietin, But Not Erythropoietin Promotes Viability and Inhibits Apoptosis of Multipotent Murine Hematopoietic Progenitor Cells In Vitro

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

The recently cloned c-mpl ligand, thrombopoietin (Tpo), has been extensively characterized with regard to its ability to stimulate the growth, development, and ploidy 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-^-^ bone marrow progenitor cells. The ability of Tpo to maintain viable Lin^-Sca-^-^ 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-^-^ progenitors 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-^-^ 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-^-^ bone marrow cells. Thus, Tpo has a potent ability to promote the viability and suppress apoptosis of primitive multipotent progenitor cells.

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Epo was kindly supplied by Boehringer Mannheim (Mannheim, Germany) and found to potentiate 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 recombiant rat (rr) SCF were kindly provided by Dr Ian K. McNiece (Ampgen Corp, Thousand Oaks, CA), rhFL, rhIL-3, and rhIL-6 were kindly supplied by ImmuneX (Seattle, WA) and rhIL-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 rhEpo, which was tested for its ability to stimulate BFU-E in combination with SCF from Lin- cells: rmTpo, 1,000 U/mL; rhEpo, 5 U/mL; rhG-CSF, 50 ng/mL; rrSCF, 100 ng/mL; rhFL, 50 ng/mL; rhIL-3, 20 ng/mL; rhIL-7, 100 ng/mL; rhIL-1α, 20 ng/mL; and rhIL-6, 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/6 mice (6 to 10 weeks old) according to previously described protocols. Briefly, B220, Ter-119, CD45, Lyt-2 (CD8), Gr-1, and Ly-6N (Gr-1), MAC-1, Lty-2 (CD8), Lyr-1 (CD5), and L3T4 (CD4; all from Pharmingen, San Diego, CA) and Ter-119 (a kind gift from Dr Tatsuo Kina, Kyoto, Japan). Cells were washed once and sheep antirat IgG (Fc)-conjugated immunomagnetic beads (Dynal, Oslo, Norway) were added at a cell:bead ratio of 1:1 (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): RA3-6B2 (B220), RB6-8C5 (Gr-1), MAC-1, Ly-2 (CD8), Lyr-1 (CD5), and L3T4 (CD4; all from Pharmingen, San Diego, CA); and Ter-119 (a kind gift from Dr Tatsuo Kina, Kyoto, Japan). Cells were washed once and sheep antirat IgG (Fc)-conjugated immunomagnetic beads (Dynal, Oslo, Norway) were added at a cell:bead ratio of 1:1 (400 × 10^6 cells/mL) and incubated at 4°C for 45 minutes on a mixing wheel. Magnetic beads were removed with a magnetic particle concentrator (MPC-6; Dynal). Unattached cells were transferred to a second tube containing the same absolute amount of magnetic beads, incubated at 4°C for 30 minutes, and processed as for the first bead separation.

Lin- cells recovered from the supernatant were further purified based on the expression of stem cell antigen-1 (Sca-1), as previously described. Briefly, Lin- cells were resuspended at 200 × 10^6 cells/mL and incubated for 20 minutes on ice with either 20 μg/mL fluorescein isothiocyanate (FITC)-conjugated rat antimonouse Ly-6A/E IgG1 antibody (Sca-1; Pharmingen) or an irrelevant FITC-conjugated purified rat IgG2b (Pharmingen) control antibody. The cells were washed and Lin- 'Sca-1' cells were sorted on a Coulter Epics Elite Cell Sorter (Coulter Electronics) equipped with a 488-nm tuned argon laser set to give a power of 15 mW, with a rate of 800 to 1,100 cells/s. Lin- cells falling into median right angle scatter and median to high forward scatter were analyzed for Sca-1 expression, and cells falling into both regions were selected. The final recovery of Lin- 'Sca-1' cells from unfraccionated BM cells was approximately 0.05%. Reanalysis of sorted Lin- 'Sca-1' cells showed reproducibly a purity of 93% to 97% and a viability of 90% to 95% as determined by propidium iodide (PI) exclusion (see below, Direct viability assay). In agreement with others, 40% ± 12% (mean of 3 experiments ± SEM) of lethally irradiated mice survived if transplanted with 100 Lin Sca-1' cells, whereas 50,000 unfraccionated BM cells resulted in 55% ± 15% survival. In some experiments, Lin- 'Sca-1' c-kit' and Lin- 'Sca-1' c-kit' cells were isolated. The same protocol as for isolation of Lin- 'Sca-1' cells was followed, except that 20 μg/mL phycoerythrin (PE)-conjugated rat antimonouse c-kit IgG2b antibody (Pharmingen) or an irrelevant PE-conjugated purified rat IgG2b (Pharmingen) control antibody was used in addition to the above-described protocol.

Phenotyping of cultured Lin- 'Sca-1' cells. In four experiments, 80,000 Lin- 'Sca-1' cells were plated in serum-depleted medium supplemented with Tpo and reexamined phenotypically for expression of lineage-specific antigens and Sca-1 after 40 hours of incubation. Cells were washed and incubated at 4°C for 20 minutes with either a cocktail of rat antimonouse antibodies (B220, Gr1, MAC-1, Ter-119, CD4, CD5, and CD8; final concentration of each antibody, 2.5 μg/mL) or irrelevant isotype-matched control antibodies (a mixture of purified rat IgG2b and IgG2a [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 (Southern Biotechnology Associates Inc, Birmingham, AL; final concentration, 10 μg/mL) at 4°C for 20 minutes. After washing, purified rat IgG (Jackson Immunoresearch Laboratories Inc, West Grove, PA) was added as a blocking reagent at 300 μg/mL (final concentration), and cells were incubated at 4°C for 5 minutes before 20 μg/mL FITC-conjugated rat antimonouse Ly-6A/E antibody (Sca-1) or an irrelevant FITC-conjugated purified rat IgG2b control 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 unfraccionated BM cells were in two experiments used as a Lin- control. In both experiments, 90% to 95% of the unfraccionated 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 Giemsa-stained.

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
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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 potently 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 as a control. Cells were counted by Trypan blue dye exclusion and 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 stimuli 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 cell number was counted by Trypan blue dye exclusion 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 enable passive diffusion of PI into dead cells through holes in 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 development further (minimum of 2 hours). Before labeling, the cells were washed once in PBS (PBS containing 1% FCS). A TdT kit (In Situ DNA 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 PBS 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 (i.e., 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\(^2\); 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 (i.e., 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,
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

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

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**Table 1. Comparison Between the Ability of Tpo and Other Putative Viability Factors to Promote Viability of Lin−Sca−1+ Progenitor Cells in Serum-Depleted Medium**

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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 ± SEM for total number of clones per 300 Lin−Sca−1+ cells of six individual experiments, with 300 wells scored in each experiment. The 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.

1 P < .01 when compared with putative viability factor alone for full incubation period.
cated concentrations of Tpo. After 40 hours of preincubation, 10 μL 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 mean ± SEM of total number of clones per 300 Lin−Sca-1+ cells of five individual experiments, with 300 wells scored in each experiment. The size distribution of clones generated from surviving Lin−Sca-1+ progenitors was similar for all concentrations of Tpo and similar to that shown in Table 1.

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 or 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
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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 of serum-depleted medium and optimal concentrations of the indicated cytokines. After 40 hours of preincubation, 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 cytospin centrifuge, fixed, and Giemsa-stained. Colonies containing more than 50% blasts (BL) were scored as a BL colony. Colonies containing more than 90% 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 Mk 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.

Fig 7. Distribution of cells produced by Lin^-Sca-1' progenitor cells surviving in response to Tpo. Lin^-Sca-1' cells were cultured at a density of 1 cell per well in 10 μL of serum-depleted medium and the indicated cytokines. After 40 hours of preincubation in Tpo, 10 μL of serum-depleted medium containing Tpo + SCF + G-CSF + IL-3 to yield predetermined optimal concentrations 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 cytospin centrifuge, fixed, and Giemsa-stained. Colonies containing more than 50% blasts (BL) were scored as a BL colony. Colonies containing more than 90% 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 Mk 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.
pressed by the viability of hematopoietic progenitor cells. Whether these c-mpl–expressing primitive progenitors include the long-term reconstituting stem cells remains to be determined.

It is possible that not all Lin-Sca-1+ progenitors responsive 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-

**DISCUSSION**

Recently Tpo, the ligand for c-mpl was cloned, and multiple studies have already shown its ability, alone and in combination with other cytokines, to potently promote the in vitro growth and differentiation of megakaryocyte progenitors 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 promote 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 reconstitution.19,21,22 It has previously been shown that some cytokines, particularly SCF, can potently promote the viability of primitive progenitors such as Lin-Sca-1+ cells in vitro while stimulating limited proliferation.19,20,23,27,30

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 medium. 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.31,37 Regardless, both the studies in FCS-supplemented and serum-depleted medium show that Tpo, when acting alone, can promote the viability of a fraction 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 lose 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 subtraction 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+ progenitor 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 include the long-term reconstituting stem cells remains to be determined.

**Table 2. The Viability-Promoting Effect of Tpo on SCF + Tpo– and IL-3 + TPO–Responsive Lin‘Sca-1’ Progenitors**

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Degree of Proliferation*</th>
<th>Total Clones/300 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preincubation</strong></td>
<td><strong>After Preincubation</strong></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>Cocktail</td>
<td>Cocktail</td>
<td>8</td>
</tr>
<tr>
<td>Tpo, SCF</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 (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 three 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 wells; 3, colonies covering 10% to 50% of well; 4, more than 50% of the well covered by cells.
TPO PROMOTES VIABILITY OF PROGENITOR CELLS

90
80
70
60
50
40
30
20
10
0

Cocktail
SCF
Tpo
Medium

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. 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. 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. In addition, Epo is a potent viability factor for committed erythroid progenitor cells. 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 growth 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-
Fig 10. Tpo counteracts apoptosis of Lin\(^{-}\)Sca-1\(^+\) BM cells. Apoptotic cells were detected using DNA strand breakage labeling with fluorescein-dUTP. (A) Thirty thousand Lin\(^{-}\)Sca-1\(^+\) cells were fixed directly after isolation or 50,000 Lin\(^{-}\)Sca-1\(^+\) cells were incubated in serum-depleted medium for 40 hours with Tpo (B) or in the absence of cytokines (C). A viable cell scatter gate was set to include greater than 90% of the freshly isolated cells (A). Cells falling within this gate were analyzed for the presence of fluorescein-dUTP labeling using a flow cytometer (FACScan). The results are from one of three experiments with similar results.

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 repleted 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 cytospin 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,\(^{16}\) 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.\(^{15,55}\) 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.52

It is widely accepted that normal hematopoiesis involves apoptosis and its regulation and that primitive hematopoietic progenitors can undergo apoptosis.4,10,26,44,45 Many cytokines prevent the apoptotic cell death of primitive progenitor cells,14,26,44,45 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,5,9,12,37,39 will remain an essential assay reflecting the viability of primitive hematopoietic progenitor cells, because direct viability/apoptosis assays do not necessarily reflect clonogenic/functional progenitors. In addition, as shown elsewhere,40,45 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 megakaryocytic 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.

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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