Thrombopoietin promotes mixed lineage and megakaryocytic colony-forming cell growth but inhibits primitive and definitive erythropoiesis in cells isolated from early murine yolk sacs

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The role of thrombopoietin (Tpo) in promoting hematopoiesis has been extensively studied in late fetal, neonatal, and adult mice. However, the effects of Tpo on early yolk sac hematopoiesis have been largely unexplored. We examined whole embryos or the cells isolated from embryo proper and yolk sacs and identified both Tpo and c-mpl (Tpo receptor) mRNA transcripts in tissues as early as embryonic day 6.5 (E6.5). Presomitive whole embryos and somite-staged yolk sacs and embryo proper cells were plated in methylcellulose cultures and treated with selected hematopoietic growth factors in the presence or absence of Tpo. Tpo alone failed to promote colony-forming unit (CFU) formation. However, in the presence of other growth factors, Tpo caused a substantial dose-dependent reduction in primitive and definitive erythroid CFU growth in cultures containing E7.5 and E8.0 whole embryos and E8.25 to 9.5 yolk sac–derived cells. Meanwhile, Tpo treatment resulted in a substantial dose-dependent increase in CFU-mixed lineage (CFU-Mix) and CFU-megakaryocyte (CFU-Meg) formation in cultures containing cells from similar staged tissues. Addition of Tpo to cultures of sorted E9.5 yolk sac c-Kit+CD34+ hematopoietic progenitors also inhibited erythroid CFU growth but augmented CFU-Mix and CFU-Meg activity. Effects of Tpo on CFU growth were blocked in the presence of a monoclonal antibody with Tpo-neutralizing activity but not with control antibody. Thus, under certain growth factor conditions, Tpo directly inhibits early yolk sac erythroid CFU growth but facilitates megakaryocyte and mixed lineage colony formation. (Blood. 2003;101:1329-1335)

Introduction

Thrombopoietin (Tpo) is a hematopoietic growth factor that stimulates the proliferation and differentiation of hematopoietic stem cells, primitive progenitors, megakaryocytes, and platelets. The cellular receptor for Tpo, c-mpl, belongs to the cytokine receptor superfamily. Early studies using antisense oligonucleotides to disrupt c-mpl function suggested that the effects of Tpo may be restricted to megakaryocytic progenitor cells. A platelet lineage-restricted action of Tpo on c-mpl-expressing cells was also observed in animals treated with Tpo. Mutant mice in which the genes for Tpo or c-mpl have been disrupted display a similar phenotype with deficient megakaryopoiesis. Both mutant strains suffer from thrombocytopenia with 100% penetrance. No substantial difference in the number of other blood cells, including red blood cells, neutrophils, lymphocytes, monocytes, and eosinophils, has been observed in either mutant strain. Expression of c-mpl has subsequently been demonstrated on hematopoietic stem cells. In vivo administration of Tpo to normal and myelosuppressed mice has also been reported to cause multilineage effects. Transplantation of c-mpl−/− mutant mice marrow into lethally irradiated recipients results in poor repopulating ability by these donor cells. Tpo has recently been found to expand hematopoietic stem cells (HSCs) in vitro as evidenced by higher levels of donor cell engraftment after transplantation into lethally irradiated mice compared with freshly transplanted marrow cells. These results have demonstrated that the effects of Tpo on hematopoiesis are more diverse than originally reported.

Although much has been learned about the role of Tpo in fetal liver and adult marrow hematopoiesis, little is known of the effect of Tpo on early yolk sac hematopoiesis. Tpo has been reported to enhance the proliferation and differentiation of erythroid cells in the presence or absence of erythropoietin in cultured embryonic day 10.5 (E10.5) yolk sac cells. In other studies, the addition of Tpo to embryonic stem cell (ES) cultures during the growth of embryoid bodies (EBs) resulted in a substantial increase in the total number of hematopoietic progenitors generated by day 6 EBs. However, the presence of Tpo in EB cultures resulted in a dramatic decrease in erythroid colony-forming unit (CFU) activity. Because the pattern and kinetics of erythropoiesis in the murine ES differentiation system largely mirror those of the murine yolk sac, the results of the 2 studies above appear to be contradictory.

We have examined the effects of Tpo on hematopoietic progenitor cell growth in vitro in cells isolated from the early murine embryo and from isolated yolk sac and embryo proper cells. We report that Tpo causes dose-dependent inhibition of primitive and definitive erythroid CFU growth but enhances mixed lineage and megakaryocytic CFU growth in vitro in cells derived from early embryos and extraembryonic yolk sacs.
Materials and methods

Mice maintenance

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Gpi/Boy mice were derived from B6Gpi-1A (gift from Dr David Harrison, Jackson Laboratories, Bar Harbor, ME) × B6.6SL-Pep3B/Boy/I (purchased from Jackson Laboratories) breeding. The F1 progeny were screened for expression of glucose phosphate isomerase 1A (Gpi-1A) enzyme activity as previously described\(^8\) and CD45.1 expression on peripheral blood leukocytes by fluorescence-activated cell sorter (FACS). To obtain timed-pregnant mice, 2 females were caged with a male overnight, and then examined for the evidence of a copulation plug the next morning. The care and use of animals in these studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Yolk sac cell preparation

Timed-pregnant mice were killed by CO\(_2\) inhalation, and the uteri were removed from the peritoneum and washed with phosphate-buffered saline or Hank's balanced salt solution (GibcoBRL, Gaithersburg, MD) several times. Embryos were obtained, and embryo proper and yolk sac were isolated as described previously.\(^8\) Presomite embryos were staged by morphologic criteria.\(^\)\(^8\) Embryos were staged by somite counting.\(^18,20\)

Dissected embryo proper or yolk sac were incubated with 0.1% collagenase/H9262 (Promega), samples were treated with 1 M diethylpyrocarbonate (DEPC) water (instead of RNA) as template were run as negative controls. RNA was isolated from yolk sac and embryo proper by using TRIzol reagent (GibcoBRL). RNA (1-5 μg) was treated with 1 μL RNase-free DNaseI (Promega, Madison, WI) at 37°C for 15 minutes, followed by heat inactivation of DNaseI at 70°C for 15 minutes to deplete DNA. The total RNA was reverse transcribed into cDNA (SuperScript for Reamplification System; GibcoBRL) according to the manufacturer’s instructions. The specific primers were used in nested PCR to amplify cDNA. Primers 1.2 and primers 3.4 were used for first- and second-round PCR, respectively. The final products were 220 bp and 100 bp for thrombopoietin (GenBank no. X73677). The primers used are as follows: Tpo primer 1, CGGGGAAGGTGCTTCCTG; Tpo primer 2, GTTTCTGAGA-CAAAATCTC; Tpo primer 3, TTCAGAGTCAGATTACTCC; Tpo primer 4, GGAAGACGGAGAATCCACC; c-mpl primer 1, CGGGAGAAG-GCGTGAGGAGGCTCAGGC; c-mpl primer 2, CTCAGGGCTGCTGGCAATAG; c-mpl primer 3, CTCAGGTCTGCAAAGTGCG; and c-mpl primer 4, CAAAGCTTGGTATGGCTAAGGAGA.

PCR amplification was 45 seconds for denaturation at 94°C, 45 seconds for annealing at 55°C for Tpo or 52°C for c-mpl, and 45 seconds for extension at 72°C for 35 cycles. Samples with diethylpyrocarbonate (DEPC) water (instead of RNA) as template were run as negative controls. The PCR fragments were visualized by ethidium bromide staining of 10 to 20 μL PCR product after electrophoresis in a 2% agarose gel. The size of PCR fragment was determined by comparing it with a 100-bp DNA ladder (Promega).

Fluorescence-activated cell sorting

The antibodies used for flow cytometry analysis were purchased from PharMingen (San Diego, CA). All antibodies were conjugated with fluorescein isothiocyanate (FITC) or allophycocyanin (APC). Rat monoclonal antibodies used in the studies included FITC-conjugated antihemoglobin g2a (IgG2a) and APC-conjugated purified rat IgG2b were used as isotype control antibodies. A single cell suspension was prepared as described in “Yolk sac cell preparation.” After centrifugation, the cell pellet was suspended in 100 μL Fc blocking antibody-conditioned medium produced by the 2A4G2 cell line (ATCC, Rockville, MD) prior to the addition of 1 μg of each specific antibody per 10\(^{6}\) cells. After 45 to 60 minutes of incubation, cells were washed with IMDM containing 10% FBS, 2% pen/strep, 2 mM glutamine, and 450 μM MTG. Cells were spun down and resuspended in the same medium for sorting, which was performed on a FACStarPLUS instrument (Becton Dickinson). C-kit−CD34\(^+\) and c-kit−CD34\(^−\) cells were collected for hematopoietic progenitor assay. In some studies, single c-kit−CD34\(^+\) cells were sorted and deposited into 96-well plates for hematopoietic progenitor assay.

Primitive erythroid colony assay

Cells were plated in duplicates or triplicates at 1 to 2.5 × 10\(^5\) cells/mL in 0.9% methylcellulose-based media (StemCell Technologies, Vancouver, British Columbia, Canada) that included IMDM, 2 mM glutamine, 1% pen/strep, 5% protein-free hybridoma medium-II (PFHM-II; GibcoBRL), 50 μg/mL ascorbic acid, 450 μM MTG, 200 μg/mL iron-saturated holo-transferrin (Sigma), 15% plasma-derived serum (Animal Technology, Antech, TX), 4 U/mL recombinant human erythropoietin (Epo; Amgen, Thousand Oaks, CA), and ± 50 ng/mL recombinant human Tpo (Peprotech, Rocky Hill, NJ). Cultures were incubated in a humidified incubator at 37°C in 5% CO\(_2\) and colonies were counted on day 7.\(^24\)

Definitive-committed progenitor assay

Cells were plated in duplicates or triplicates at 1 to 2.5 × 10\(^5\) cells/mL in 0.9% methylcellulose-based media that included IMDM, 2 mM glutamine, 1% pen/strep, 10⁻² M β-mercaptoethanol, 30% fetal bovine serum, 4 U/mL recombinant human Epo (Amgen), 100 U/mL recombinant murine interleukin-3 (IL-3; Peprotech), 100 ng/mL recombinant murine stem cell factor (SCF; Peprotech), and ± 50 ng/mL recombinant human Tpo (Peprotech). Cultures were incubated in a humidified incubator at 37°C in 5% CO\(_2\) in air, and colonies were counted on day 7,22,25

CFU-Meg and BFU-Meg assay and immunohistochemical staining

Cells were plated in duplicates or triplicates at 1 to 2.5 × 10\(^5\) cells/mL in 0.3% agar-based McCoy 5A medium (GibcoBRL)\(^2\) that included 10% fetal bovine serum, 100 U/mL recombinant IL-3, and ± 50 ng/mL recombinant human Tpo. Cultures were incubated in a humidified incubator at 37°C in 5% CO\(_2\) in air. After 7 days (for CFU, megakaryocyte [CFU-Meg]) or 14 days (for burst-forming unit, megakaryocyte [BFU-Meg]) incubation, these 35-mm grid culture dishes were air dried over night and fixed with 1:3 methanol/acetone (Fisher, Fair Lawn, NJ). Fixed cultures were rehydrated in pH 7.6, 0.05 M Tris (tris(hydroxymethyl)aminomethane)/0.15 M NaCl buffer for 20 minutes, followed by additional 0.5 mL 5% mouse serum for 20 minutes. Primary antibody (10 μg/mL rat antimaouse CD41; PharMingen) or rat IgG \(\kappa\) isotype control antibody (5 μg/mL rat IgG2a; Pharmingen) in Tris/NaCl buffer with 5% mouse serum was added to the cultures and incubated for 30 minutes. After washing, secondary antibody (10 μg/mL biotinylated antirat IgG; Vector, Burlingame, CA) was added and incubated for 30 minutes, followed by 18 μg/mL alkaline phosphatase streptavidin (Vector) for 30 minutes. Plates were washed and subjected to alkaline phosphatase substrate (Vector) according to the manufacturer’s instruction. Gently rinsed plates and colonies were scored after drying. CFU-Megs and BFU-Megs were scored according to their colony morphology and CD41 expression.
Neutralizing antibody-blocking studies

Antimurine Tpo-neutralizing antibody or antihuman Tpo-neutralizing antibody or isotype control antibody (Peprotech) was incubated with murine Tpo or human Tpo (Peprotech) at 37°C for 1 hour, respectively. Then, the mixture of antibody and cytokine was added to the regular culture medium for primitive erythroid, definitive-committed progenitor, and megakaryocyte assays and plated as described earlier.

 Colony morphology analysis

CFU Mixed lineage (CFU-Mix) from definitive-committed progenitor assay were plated and digested in 50 μL 0.25% trypsin (StemCell Technologies) for 2 minutes and diluted in 100 μL IMDM with 10% serum. The single cell suspension was added to 100 μL 10% bovine serum albumin (BSA; Ortho Clinical Diagnostics, Paritan, NJ) and centrifuged onto glass microscope slides at 500 rpm in a Cytospin (Shandon, England) device for 5 minutes. After air-drying, slides were fixed and stained with Diff-Quik Stain Set (Dade Behring, Dudingen, Switzerland). Cells were scored morphologically under oil-immersion light microscopy.

 G1E-ER2 erythroid cell line

G1E-ER2 cells have been previously described.27-29 These cells were grown in IMDM (Invitrogen) with 15% heat-inactivated fetal bovine serum (Fisher), recombinant Epo (2 U/mL) (Amgen), and recombinant mouse (rm) SCF (50 ng/mL) (Amgen).

 Apoptosis analysis on G1E-ER2 cells

G1E-ER2 cells were starved of growth factors and serum for 5 hours and cultured for 48 hours in the presence of 50 ng/mL SCF or Tpo or 2 U/mL Epo. Apoptosis was measured by staining cells with Annexin V and analyzed by flow cytometry. Briefly, cells were resuspended in 100 μL Annexin V, Cells were then vortexed and incubated for 15 minutes at room temperature. Then, an additional 400 μL binding buffer was added, and cells were analyzed by flow cytometry as described before.28

 Immunoprecipitation

G1E-ER2 cells were starved for 6 hours at 37°C and stimulated with 500 ng/mL Tpo for indicated times or left unstimulated. Cells were lysed and equal amount of protein was subjected to immunoprecipitation by using an antimurine Mpl antibody (kindly provided by Frederic J. de Sauvage, Genetech, South San Francisco, CA), and Western blot analysis was performed with an antiphosphotyrosine antibody as described previously.28

 Statistical analysis

Data are expressed as the mean ± SEM where applicable. Differences between groups were analyzed by means of a nonparametric Mann-Whitney test. A probability value of < .05 was considered significant. All experiments were performed in triplicate on 2 to 4 experiments.

Results

Tpo and c-mpl are expressed in early embryonic development

We examined early whole embryos for Tpo and c-mpl mRNA expression. For E6.5 and E7.0 embryos, the whole embryos were used for RNA extraction. For E8.0 and later embryos, we dissected yolk sac and embryo proper tissues for RNA extraction and nested RT-PCR analysis. A 220-bp nucleotide PCR product representing Tpo and a 368-bp nucleotide product representing c-mpl were observed in E6.5 and E7.0 whole embryo tissue (Figure 1). Similar products were present in both yolk sac and embryo proper cells isolated on E8.0, E9.0, E10.0, E11.0, E13.0, and E14.0. These results indicated that both Tpo and c-mpl mRNA are expressed throughout the hematopoietic phase of yolk sac development and in the early embryo during the period of initiation of definitive hematopoiesis.

The presence of mRNA for these molecules in the gastrulating embryo caused us to question whether Tpo and c-mpl were expressed prior to gastrulation. We isolated mRNA from murine blastocysts and an embryonic stem cell line (R1) for RT-PCR analysis and identified both Tpo and c-mpl mRNA transcripts (X.X. and B. Chen, unpublished observations, September 2002). These results indicate that Tpo and c-mpl are expressed in pluripotent cells prior to and during gastrulation. This early appearance of Tpo and c-mpl message predates the emergence of not only megakaryocytes but also of all hematopoietic elements in both the yolk sac and embryo proper.

Tpo diminishes primitive and definitive erythroid CFU formation but enhances mixed lineage and megakaryocyte CFU enumeration

To determine the effect of Tpo on early hematopoietic progenitor formation in vitro, we examined primitive and definitive erythroid CFU growth. In E7.5 whole embryos, only primitive erythroid CFU (EryP) can be detected (Figure 2A). In E8.25 whole embryo and E8.5 yolk sac, both primitive and definitive CFU cells can be detected (Figure 2B-C). In the E9.5 yolk sac, primitive erythroid CFUs had disappeared and only definitive CFUs were enumerated in culture (Figure 2D). The presence
of Tpo in the culture system resulted in a decreased number of EryPs in cultured E7.5 and E8.25 whole embryo and E8.5 yolk sac and embryo proper cells (Figure 2A-C). Tpo also decreased the output of definitive erythroid CFU cells (BFU-E) in E8.25 whole embryo and E8.5 and E9.5 yolk sac cell cultures (Figure 2B-D).

However, the presence of Tpo in the same cultures augmented the number of CFU-Mix cells enumerated in E8.25 whole embryo and E8.5 and E9.5 yolk sac cell cultures (Figure 2B-D). As anticipated, Tpo increased the production of both CFU-Meg and BFU-Meg cells in E8.25 whole embryo and E8.5 and E9.5 yolk sac cell cultures (Figure 2B-D). We also identified colonies in cultures containing E8.5 and E9.5 embryo proper cells. Although the number of CFUs enumerated was much lower than that of yolk sac (Table 1), Tpo diminished BFU-E output by 50%, had no effect on CFU-Mix, and increased CFU- and BFU-Megs by 2-fold.

**Dose-dependent effects of Tpo on hematopoietic progenitor formation in vitro**

To further confirm the above results, we examined the dose-dependent effects of Tpo on different progenitor compartments, including EryP, BFU-E, CFU-Mix, CFU-GM, and CFU-Meg in cells isolated from these early embryos. Except for CFU-GM, all CFUs responded to added Tpo in a dose-dependent fashion. BFU-Es and EryP CFUs decreased in a reciprocal fashion as the concentration of Tpo increased in the cultures containing E8.5 or E9.5 yolk sac cells (Figure 3A-B). On the contrary, CFU-Mix and CFU-Meg frequencies linearly increased with increasing concentrations of added Tpo (Figure 3B). Therefore, colony formation of EryP, BFU-E, CFU-Mix, and CFU-Meg are responsive to Tpo over a wide range of concentrations, whereas CFU-GM appeared unresponsive (data not shown).

**Effect of anti-Tpo–neutralizing antibody on Tpo modulation of CFU formation**

To further substantiate that the effects of Tpo on hematopoietic progenitor formation are a direct effect of Tpo, we examined the consequences of the addition of an antibody that binds to and neutralizes Tpo action by blocking binding of Tpo to c-mpl. Because of the endogenous expression of Tpo and c-mpl, we first confirmed that addition of the antimurine Tpo-neutralizing antibody (anti-mTpo) to cultures had no effect on baseline CFU formation (Table 2).

We noted no change in the number of hematopoietic progenitors cultured in the presence or absence of the anti-mTpo–neutralizing antibody, suggesting that endogenous murine Tpo present in the early embryo is not playing a substantial role in the methylocellulose cultures on plating of yolk sac or embryo proper cells. Adding antihuman Tpo (anti-hTpo)–neutralizing antibody to the culture system in the presence of human (hTpo) blocked the previously observed hTpo-induced inhibition of EryP colony formation. This resulted in an observed increase in the number of EryP CFUs compared with cultures with hTpo but no added neutralizing antibody. Increasing concentrations of neutralizing antibody completely rescued EryP colony numbers in the presence of hTpo, whereas a control antibody did not show any effect (Figure 4A). Similarly, BFU-E colony numbers were substantially increased by adding anti-hTpo–neutralizing antibody in hTpo-supplemented cultures to the level approaching those cultures containing no hTpo (Figure 4B). However, anti-hTpo–neutralizing antibody decreased the number of BFU-Mixs and CFU-Megs in cultures with added

**Figure 4. The inhibitory effect of hTpo on erythroid and the positive effect of hTpo on CFU-MIX and CFU-Meg are blocked by adding neutralizing anti-hTpo antibody in the cultures.** E8.5 yolk sac cells were plated at 2500 cells/250 μL in 24-well plates in the primitive erythroid colony assay containing various concentrations of neutralizing anti-hTpo antibody or control antibody (A). E9.5 yolk sac cells were plated at 10 000 cells/mL in the progenitor assay or megakaryocyte assay containing 0.5 μg/mL neutralizing anti-hTpo antibody or control antibody (B). The statistical significance when various conditions are compared with the condition of no Tpo, P < .05. **The statistical significance when hTpo + anti-hTpo group is compared with that of hTpo and hTpo + control antibody, P < .05. Data represent the mean values of triplicate samples from 4 experiments; error bars represent SEM.
In clonogenic assays. We interpret these results to indicate that the effects of Tpo on hematopoietic progenitors is not dependent on the presence of a Tpo-responsive accessory cell type that is secreting unknown factors to inhibit erythroid and augment mixed lineage and megakaryocyte CFU formation.

To confirm the effect of Tpo on sorted cells, we performed a single cell analysis of progenitor cell formation in vitro. In 3 experiments, single c-kit+CD34+ cells from E9.5 yolk sac were sorted into 96-well plates in the presence or absence of Tpo. This assay is more informative because it compares all lineage readouts under identical in vitro conditions. Representative data from one of 3 experiments (CFU-plating efficiency is 15% with 15.4% of single cell deposition and 10.6% of standard CFU assay) revealed that the number of BFU-Es cloned was 5 of 373 cells plated in the presence of Tpo and 13 of 374 cells plated in the absence of Tpo, whereas the number of CFU-Mixes cloned was 34 of 373 and 33 of 374, respectively. Tpo had no apparent effect on the number of BFU-Es, as the number of CFCs cloned was 15 of 373 cells plated in the presence of Tpo and 15 of 374 in the absence of Tpo. The number of megakaryocyte-containing CFCs was 10 of 373 cells plated in the presence of Tpo and 4 of 374 in the absence of Tpo. Although the effect of Tpo was not apparent on CFU-Mix formation in these experiments, the effects of Tpo on BFU-E and megakaryocyte colonies was similar to the results obtained when c-kit+CD34+ yolk sac-derived cells were plated in a bulk progenitor assay and supports a direct effect of Tpo on these progenitor cells.

**Effect of Tpo on late yolk sac and adult marrow CFU**

Given the evidence that Tpo has a positive effect on E10.5 yolk sac and adult marrow erythroid CFU formation, we also examined the effect of Tpo on late yolk sac and adult marrow progenitors under our culture conditions. In E10.5 embryo proper (Figure 5A), few colonies were identified; a finding consistent with previous studies. Remarkably, more colonies were found in plated E10.5 yolk sac cell cultures (Figure 5B). Tpo did not alter BFU-E and CFU-Mix formation, but CFU-Meg formation substantially increased (Figure 5B).

To further characterize the lack of effect of Tpo on BFU-E formation at the functional and biochemical level, we used an ES cell–derived erythroid progenitor cell line, G1E-ER2. These cells were derived from in vitro differentiated GATA-1−/− ES cells and represent primary GATA-1−/− erythroblasts as determined by the expression of erythroid but not myeloid genes. Remarkably, restoration of GATA-1 function in these cells restores erythroid maturation. Using these cells, we examined the effect of Tpo in augmenting or inhibiting cell survival and proliferation, alone, or in the presence of SCF and Epo. As seen in Figure 6B, despite substantial phosphorylation of c-mpl in response to Tpo stimulation, Tpo alone or in the presence of SCF or Epo did not substantially modulate erythroid cell survival (Figure 6A) or proliferation (data not shown). These results demonstrate that, despite the presence of a functional c-mpl receptor, SCF or Epo fails to augment or inhibit the effects of Tpo on erythroid cells.

![Figure 5. Effect of Tpo on E10.5 embryo, yolk sac, and adult bone marrow BFU-E, CFU-Mix, CFU-GM, and CFU-Meg.](image-url)
These results are consistent with our results in primary late yolk sac cultures and suggest that Tpo might differentially regulate erythropoiesis at different stages of erythroid cell development. Tpo also increased BFU-E formation in plated adult bone marrow cells (Figure 5C). Consistent with the lack of a Tpo effect on CFU-GMs in early murine cells, Tpo did not change CFU-GM formation in plated E10.5 yolk sac or adult marrow cells. Thus, the effect of Tpo on early yolk sac hematopoietic cells varies from the effect of Tpo on late yolk sac, adult marrow, or cultured erythroblast cells.

**Discussion**

We have demonstrated the presence of Tpo and c-mpl mRNA transcripts in pregastrulating embryonic cells and in yolk sac and embryo proper cells during early embryogenesis. We have also provided evidence that Tpo inhibits erythroid colony formation in vitro in cells isolated from presomite-staged embryos and yolk sacs but augments megakaryocyte lineage (CFU-Meg and BFU-Meg) and mixed lineage CFU cell formation from these same tissues. These data support a growing body of literature suggesting that the effects of Tpo on hematopoiesis are multineigline and highlight differences in the response of hematopoietic progenitors to growth factors at different stages of ontogeny.

Murine hematopoiesis arises in yolk sac blood islands and becomes morphologically identifiable at E7.0 to 7.5. At first, primitive erythroid progenitors comprise nearly all blood island progenitors; however, definitive progenitors begin to emerge at E8.25. We have investigated the temporal emergence of Tpo and c-mpl mRNA transcripts and examined the biologic responsiveness of early yolk sac and embryo proper hematopoietic progenitors to pharmacologic doses of Tpo in vitro. On the basis of our RT-PCR analysis, the transcripts of Tpo and c-mpl were present prior to the appearance of the first yolk sac blood islands and throughout the hematopoietic phase of embryo development. Because blood cells start to emerge on E7.0 to 7.5, we isolated embryos at this stage and later to determine if hematopoietic progenitors were Tpo responsive. In E7.5 embryos and older, we found 2 waves of detectable progenitors: primitive erythroid and definitive progenitors. The first wave of primitive erythroid CFUs emerged on E7.5 but were undetectable at E9.5 in either the yolk sac or embryo proper. The second wave of definitive progenitors was first identifiable on E8.25. These findings are consistent with those published by Palis et al who used a combination of 11 growth factors to identify primitive erythroid progenitors on E7.0 of gestation and emergence of definitive progenitor cells in the yolk sac on E8.25. From E8.5 and later in the present studies, isolated embryos were dissected into embryo proper and yolk sac, and we identified definitive CFU cells in both yolk sac and embryo proper. At all stages, the frequency of each CFU lineage in the yolk sac exceeded those found in the embryo proper. Our data support the results of Palis et al who speculated that the definitive hematopoietic progenitors emerge predominantly in the yolk sac and subsequently enter the circulation for colonization of the fetal liver.

We identified a dose-dependent inhibitory effect of Tpo on both primitive and definitive erythroid CFU formation. In contrast, Tpo augmented megakaryocyte lineage (CFU-Meg and BFU-Meg) and CFU-Mix formation in a dose-dependent manner. These apparent direct effects of hTpo on hematopoietic progenitors were confirmed by anti-hTpo-neutralizing antibody studies. When cultures containing hTpo were supplemented with anti-hTpo-neutralizing antibody, the biologic effects of the growth factor were abrogated in every experimental group. Interestingly, the inhibitory effect of hTpo on BFU-E formation was totally rescued by adding 0.5 µg/mL anti-hTpo-neutralizing antibody in the hTpo-supplemented culture system (Figure 4B). However, the inhibitory effect of hTpo on EryP formation was not totally rescued by using the same concentration of neutralizing antibody (Figure 4A). At the same time, the positive effect of hTpo on CFU-Mix and CFU-Meg formation was also not totally abolished by 0.5 µg/mL anti-hTpo-neutralizing antibody (Figure 4B). These observations may be explained by varying sensitivities of different progenitors to Tpo.

We did not observe an effect of Tpo on E10.5 yolk sac erythroid progenitor cells, as previously noted by Era et al. This observation may be explained by the different serum and cytokines that we used in our study. We also failed to see any effect of Tpo on our erythroblast cell line, despite the evidence of c-Mpl receptor activation. We did observe a positive effect of Tpo on BFU-E formation in plated adult marrow cells. This effect is similar to that observed when mice deficient in Tpo expression (Tpo−/− mice) were treated with exogenous Tpo. Although the lack of Tpo (Tpo−/−) resulted in diminished BFU-E, CFU-Mix, CFU-, and BFU-Meg progenitor cell numbers, all of these progenitor compartments were rescued to greater than wild-type control levels when exogenous Tpo was administrated in vivo. These results indicate that Tpo differentially affects erythroid CFU formation, depending on the stage and site of hematopoietic development in the mouse, whereas the effects of Tpo on CFU-Mix and CFU- and BFU-Meg are similar throughout murine ontogeny.

There are several possibilities to explain the mechanism through which Tpo simultaneously inhibits erythroid but augments mixed lineage and megakaryocyte CFU formation in the early embryo. First, Tpo might send an inhibitory signal to erythroid precursors but a growth progression signal to mixed lineage and megakaryocyte progenitors directly or indirectly, although whether this is true or not needs to be further studied. As noted earlier, we did not observe a Tpo-induced apoptotic response in cultured erythroid cells. Alternatively, similar to the in vitro study of ES cell-derived hematopoiesis, the idea of bipotent clonogenic precursor for both erythroid and megakaryocyte may explain what we observed. According to this theory, the cell may respond to Tpo by preferentially differentiating into mixed lineage and megakaryocyte progenitors at the expense of erythroid progenitors.
Because of the different kinetics of erythropoiesis and megakaryocytogenesis (a common precursor may give rise to a number of erythroid progenitors but just a few megakaryocyte progenitors), comparing CFU growth in early yolk sac and embryo proper cells in vitro. It remains unclear if Tpo plays a substantial role in steady-state hematopoiesis in the yolk sac in vivo. Detailed analysis of yolk sac hematopoiesis in existing mutant mice in which Tpo or c-mpl have been disrupted may be informative. Such variation in the response of early and late yolk sac and adult marrow cells to Tpo is similar to previously reported differences in growth factor responsiveness of embryonic versus fetal and adult mouse progenitor cells treated with Epo or SCF and highlights how differences in the hematopoietic microenvironment influence progenitor cell behavior.

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