Rapid Publication

Thrombopoietin Expands Erythroid Progenitors, Increases Red Cell Production, and Enhances Erythroid Recovery after Myelosuppressive Therapy

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Abstract

Thrombopoietin (TPO), the ligand for the receptor protooncogene c-*mpl*, has been cloned and shown to be the critical regulator of platelet production. Several features of c-Mpl expression, including its presence on erythroid cell lines, and the panmyeloid transformation characteristic of myeloproliferative leukemia (MPL) viral disease led us to investigate whether this receptor–ligand system may play a role in erythropoiesis. We report that although TPO alone did not support the growth of either early or late erythroid progenitors, it acted in synergy with erythropoietin to expand these populations. Moreover, while the effects on erythropoiesis in normal animals were modest, TPO greatly expanded the number of erythroid progenitors and blood reticulocytes and was associated with accelerated red cell recovery in myelosuppressed mice. Together, these data strongly suggest that erythroid progenitors respond to TPO and that this newly cloned cytokine, critical for platelet production, can augment erythropoiesis in states of marrow failure. (*J. Clin. Invest.* 1995. 96:1683–1687.) Key words: thrombopoietin • erythropoiesis • c-Mpl • myelosuppression

Introduction

Recently, we and others have cloned the ligand for the c-Mpl receptor (1–4). In subsequent studies it was shown that the Mpl ligand supports the proliferation of megakaryocytic progenitors and their differentiation into large polyploid, platelet-producing megakaryocytes (5, 6). Moreover, Mpl ligand levels are inversely related to platelet count, and the administration of the cytokine profoundly drives platelet production in normal animals (5, 6). On these grounds, we proposed that Mpl ligand

is identical to thrombopoietin (TPO),[1] the critical regulator of thrombopoiesis (7, 8). A more recent study in which Mpl receptor expression was eliminated by homologous recombination in genetically engineered mice (9) substantiates this conclusion.

The regulation of red blood cell (RBC) development is one of the critical homeostatic mechanisms of higher organisms. Over the past several years, many of the humoral regulators of erythropoiesis have been identified and molecularly cloned. Although IL-3 (10, 11), GM-CSF (12, 13), and c-*kit* ligand (KL [14]) have been shown to expand early erythroid progenitors, erythropoietin (Epo), which promotes the terminal differentiation of these cells, is thought to be the critical regulator of red cell production (15, 16).

In recent years, multiple lines of evidence point to a common origin of the erythroid and megakaryocytic lineages (for review see reference 17). For example, erythroleukemic cell lines express markers of megakaryocytic differentiation (18–20), and erythroid and megakaryocytic cells display a number of common surface markers and transcription factors (21, 22). Furthermore, several groups have shown that Epo augments the effects of plasma TPO on megakaryocyte formation in vitro, although the platelet response to its in vivo administration to normal animals has been variable (23–25). To determine the effects of TPO on erythropoiesis we evaluated the effects of this new cytokine on in vitro and in vivo red cell production, using both normal mice and those rendered pancytopenic with myelosuppressive therapy. We report that in Epo-containing cultures, TPO enhances the development of early erythroid progenitors and the generation of late erythroid colony–forming cells. In vivo, after cytoreductive therapy, TPO speeds the repopulation of the marrow and spleen with both early and late erythroid precursors, resulting in enhanced red cell production and earlier erythrocyte recovery. These results strongly support the hypothesis that erythroid and megakaryocyte progenitors respond to overlapping signals and suggest that the clinical utility of thrombopoietin may be greater than initially anticipated.

Methods

In vivo studies. 8–12-wk-old female Balb/c mice (The Jackson Laboratories, Bar Harbor, ME) were maintained in a controlled American Association for Laboratory Animal Care–certified animal care facility and provided water and standard laboratory chow ad lib. All procedures were approved by the University of Washington and ZymoGenetics Animal Care Committees. For studies in normal mice, groups of five animals were administered recombinant murine (rm) TPO intraperitoneally once daily and killed on day 7. Additional groups of five mice were exposed to 350 cGy total body irradiation from a ^{137}Cs source

(Gammacell 40 Irradiator; Atomic Energy of Canada Radiochemical Co., Kanata, Canada) followed by a single intraperitoneal injection of 1.2 mg carboplatinum, adapting a previously described model (26). On the following day, treatment was begun with either vehicle or rmTPO, subcutaneously twice daily. Blood counts were performed on 40-μl samples obtained by the retroorbital route, using a hematology analyzer (9010; Baker-Serono, Allentown, PA) set for murine parameters, except where otherwise indicated. Reticulocyte indices were obtained by visual inspection of > 1,000 red cells per sample after supra vital staining or by flow cytometric analysis. At death, necropsy of the lungs, liver, brain, and intestinal tract was performed noting macroscopic or microscopic signs of blood loss.

Progenitor cell assays. Total early erythroid progenitors (burst-forming unit-erythroid [BFU-E]) were quantitated in the presence of pokeweed mitogen spleen cell–conditioned medium (PWM-SCM) and Epo. Cells from the marrow or spleen were cultured in 1.4% methylcellulose in Iscove's Modified Dulbecco's medium supplemented with 30% FCS, 1% BSA, 5×10^{-5} M β-mercaptoethanol, recombinant human erythropoietin (2 U/ml), and 1% PWM-SCM (Stem Cell Technologies, Vancouver, Canada) in a humidified atmosphere at 37°C. Erythroid bursts were counted on day 8. Late erythroid colonies (CFU-E) were grown in plasma clots in the presence of 0.5 U/ml Epo (27). To determine the effects of individual cytokines on the growth of BFU-E–derived colonies, recombinant IL-3 (obtained as the serum-free culture medium of baby hamster kidney [BHK] cells engineered to express a full-length IL-3 cDNA [28]) or recombinant murine KL (Genzyme Corp., Boston, MA) were used in place of PWM-SCM. To test for the generation of late erythroid progenitors, a two-phase culture system was used. In the first stage, normal BDF_1 (C57BL/6 $\times$ DBA/2 F_1) murine marrow cells were adhered to plastic tissue culture flasks for 2 h to deplete macrophages. The nonadherent cells (5×10^5/ml) were plated in Iscove's modified Dulbecco's medium supplemented with 1% Nutridoma medium (Boehringer-Mannheim Corp., Indianapolis, IN), 5% FCS, and with combinations of purified cytokines. After 2, 4, 7, and 9 d of culture, aliquots of cells were removed and CFU-E quantitated using standard conditions (27). Megakaryocytic progenitor cell numbers were determined as previously described (5). Marrow cells were obtained by flushing both femurs into CATCH medium (0.38% sodium citrate, 2×10^{-3} M theophylline, 1×10^{-3} M adenosine in HBSS) and were plated at 2×10^5 per ml in Iscove's modified Dulbecco's medium supplemented with 15% preselected horse serum, antibiotics, 600 U/ml murine IL-3, and 1,000 U/ml murine TPO and were made semisolid with 0.275% agar. Cultures were incubated in a fully humidified 5% CO_2-containing atmosphere, and megakaryocyte colonies (containing $\geq$ 3 cells) were enumerated by inverted microscopy on day 5.

Recombinant TPO. The rmTPO used for in vivo studies was expressed in mammalian cells and assayed as previously described (1). Conditioned media were collected and concentrated by ultrafiltration. An affinity column was constructed by coupling murine Mpl receptor to CNBr Sepharose and was used to purify rmTPO. After buffer exchange to 20 mM Tris/1 mM EDTA, the protein was loaded onto the column and eluted with 3 M KSCN. Fractions containing material absorbing at A_{280} were pooled and dialyzed into 20 mM Tris (pH 8). The final material consisted of a mixture of TPO molecular weight forms with the 70- and 30-kD forms most prominent. Doses are expressed as units, where 50 U are defined as the amount of rmTPO stimulating a half-maximal response in the BaF3/mouse mpl mitogenesis assay (1, 5). For in vitro experiments, serum-free culture medium conditioned by BHK cells engineered to express the full length form of rmTPO was used at appropriate dilution. Control experiments contained a similar dilution of sham-transfected BHK cell serum-free culture medium. In three in vitro experiments, highly purified rmTPO was used.

Statistics. Significant differences between paired results were determined by the Mann-Whitney test.

Results and Discussion

In initial studies, normal mice were treated for 7 d with either vehicle or rmTPO, and their hematologic parameters were moni-

Table I. Erythropoiesis in Normal Mice Treated with TPO

	CFU-Mk	BFU-E	CFU-E	Reticulocytes	RBC
	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-5}$	%	$\times 10^{-6}/mm^3$
Vehicle					
Femur	0.6±0.09	1.1±0.3	1.7±0.2	—	—
Spleen	2.1±0.5	19±4.0	1.2±0.2	—	—
Blood	—	—	—	8.7±0.3	10.2±0.2
TPO					
Femur	14±0.1*	2.6±0.5*	1.0±0.1*	—	—
Spleen	46±5.7*	24±2.5	3.0±0.4*	—	—
Blood	—	—	—	12.0±0.3*	9.7±0.07

9-wk-old male Balb/c mice were treated daily with vehicle or 18 kU rmTPO for 7 d, administered as a single intraperitoneal injection, and marrow and splenic cells were harvested. The results represent the mean number of colonies±SEM per organ from five vehicle and five TPO-treated animals determined from triplicate plating. The results for CFU-megakaryocyte (*CFU-Mk*) were previously reported (5) and are shown for comparative purposes. In this experiment, reticulocytes were determined by flow cytometry and red blood cell (RBC) counts by Coulter counter in the University of Washington clinical hematology laboratory. Similar results have been obtained in three additional experiments using BDF_1 mice. $* P < 0.05$, determined by two-tailed Mann-Whitney test.

tored (5). Peripheral blood platelet counts increased from a mean of $1.2\pm0.02 \times 10^6/mm^3$ in control animals to $4.4\pm0.4 \times 10^6/mm^3$ in the TPO-treated mice due to a 20-fold increase in megakaryocytic progenitors and a 9-fold increase in marrow megakaryocytes. Marrow and spleen cells were also tested for the number of early (BFU-E) and late (CFU-E) erythroid precursors under optimal culture conditions. Compared to mice treated with vehicle alone, BFU-E were significantly expanded in the marrow and CFU-E redistributed from the marrow to the spleens of TPO-treated animals. As indicated by the increased reticulocyte counts in the TPO-treated mice, these changes in erythroid progenitors were associated with a minor increase in the rate of red cell production (Table I). Despite these changes, however, RBC levels in the TPO-treated animals failed to rise. The modest effect of TPO on erythropoiesis in our experiments with normal animals may have been due to the short duration of exposure, the low levels of other cytokines critical for erythroid development, or the action of counterregulatory mechanisms which temper responsiveness. This latter mechanism was invoked to explain the differences between the effects of IL-3 on neutrophil production in normal and pancytopenic primates (29).

As part of a study to establish the effectiveness of TPO in thrombocytopenic animals and to determine if its effects on erythropoiesis might be more pronounced if potential restraints on erythropoiesis were eliminated, we administered TPO to myelosuppressed mice. The effects of TPO on megakaryocytic progenitors and platelet recovery were marked and will be reported elsewhere (Grossmann, A., J. Lenox, J. M. Humes, H. P. Ren, J. W. Forstrom, K. Kaushansky, and K. H. Sprugel, manuscript submitted for publication). In addition, compared to animals receiving vehicle alone, the magnitude of the drop in red blood cell levels was significantly reduced, and recovery was accelerated (Fig. 1). Nadir red blood cell counts were 41% of normal in vehicle-treated animals compared to 80% of normal
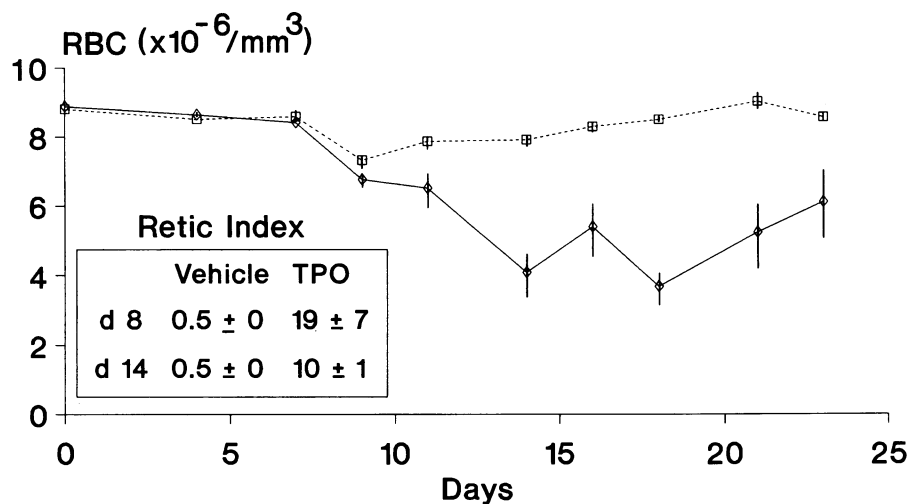
RBC (x10^{-6}/mm^3)

Retic Index

	Vehicle	TPO
d 8	0.5 ± 0	19 ± 7
d 14	0.5 ± 0	10 ± 1

Days

Figure 1. TPO improves red cell recovery after myelosuppressive therapy. 8-wk-old female Balb/c mice were given a sublethal dose of total body irradiation followed by a single intraperitoneal injection of carboplatinum on day 0. Red cell and reticulocyte levels were measured on samples collected by retroorbital bleeding before myelosuppression and at the indicated days after irradiation. The data represent the mean±SEM red blood cell counts of five mice treated with vehicle (—◇—) or five mice treated with 50 kU rmTPO (--⊟--), injected subcutaneously twice daily on days 1–15. All results from days 11 to 23 are significant at $P < 0.05$ level by Mann-Whitney test. Similar results were obtained in four additional experiments.

in TPO-treated mice. In these animals, no macroscopic or microscopic signs of bleeding were noted. Moreover, red cell production in the TPO-treated animals was enhanced, manifest by increased reticulocyte counts during the recovery period.

To further investigate the mechanism of the protective effects of TPO on red cell levels after myelosuppressive therapy, we performed additional experiments in which multiple erythropoietic parameters were monitored throughout the recovery period. Mice were treated with radiation and carboplatinum and

then administered either rmTPO or vehicle for 12 d and killed on day 13. Although microscopic bleeding was noted on the surface of the brain and at an occasional site in some of the vehicle-treated animals in this particular experiment that was not detected in the TPO-treated mice, the latter group also displayed profound changes in red cell production (Fig. 2). The mechanism of increased erythrocyte production was an expansion of marrow and splenic erythroid precursors. Although hematopoietic progenitors of all types were barely detectable in the myelosuppressed vehicle-treated animals, in myelosuppressed TPO-treated mice early erythroid progenitors had recovered to 20% of the level present in normal animals, late erythroid progenitors had recovered to 60%, and blood reticulocyte levels had recovered to those found in normal animals. These changes
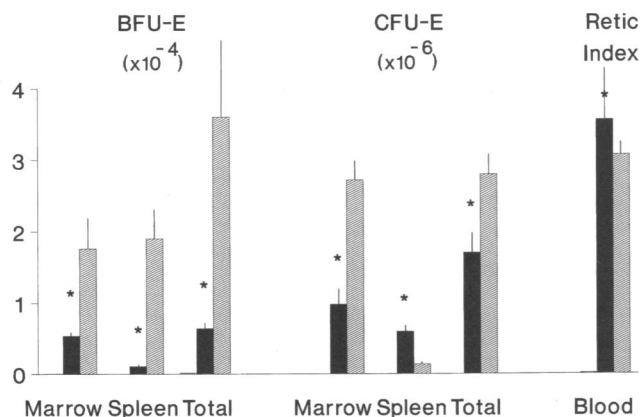

Figure 2. TPO hastens erythroid progenitor recovery after myelosuppressive therapy. Balb/c mice were treated with radiation and carboplatinum (as described in Fig. 1) and then with either vehicle (□) or 40 kU/d rmTPO (■) given subcutaneously twice daily for 12 d. Midway through the platelet recovery phase (day 13) the animals were killed and marrow and spleen obtained and assayed for all early and late erythroid progenitors. The results represent the mean progenitor numbers (±SEM) of five animals in each group. The reported progenitor numbers are for the entire marrow, assuming that one femur represents 6% of the total marrow-bearing space (30). The total number of progenitors is the sum of those present in the marrow and spleen. In contrast to erythroid progenitor levels of 10–65% of normal values in myelosuppressed, TPO-treated animals, virtually no erythroid progenitors were detected in the myelosuppressed, vehicle-treated mice. Similar results were obtained in two additional experiments in C57B1/6 mice given once-daily doses of vehicle or rmTPO. The values from five age- and sex-matched normal Balb/c mice ([▨]) are shown for comparison. Significant differences (*) between vehicle and TPO-treated myelosuppressed animals were determined by the Mann-Whitney test.
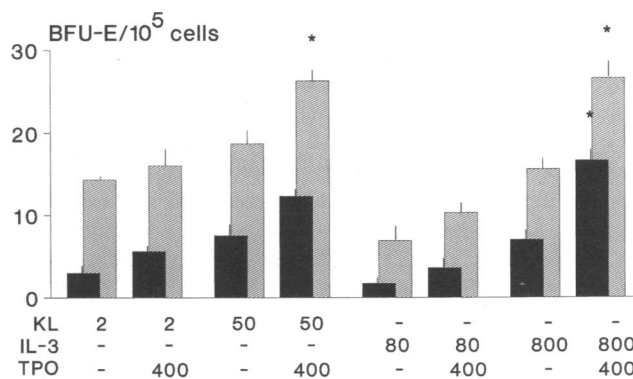

BFU-E/10^5 cells

KL	2	2	50	50	–	–	–	–
IL-3	–	–	–	–	80	80	800	800
TPO	–	400	–	400	–	400	–	400

Figure 3. TPO augments the size and number of erythroid bursts in response to IL-3 or KL and Epo. Early erythroid progenitors (BFU-E) were assayed as described in Methods using purified cytokines instead of PWM-SCM. All cultures contained 2 U/ml recombinant human Epo, added on day 3. Cultures containing 80 or 800 U/ml rmIL-3, 2 or 50 ng/ml rmKL, sham BHK-conditioned medium or 400 U/ml rmTPO are indicated. The data ([▨]) represent the mean total number of BFU-E (±SEM) of quadruplicate plates from a representative experiment. The number of large bursts, containing > 10,000 cells as determined by inverted microscopy, are indicated by solid bars (■). Similar results were obtained in four additional experiments, two of which used purified rmTPO. Statistically significant differences (Mann-Whitney test) between cultures grown in the absence or presence of TPO are indicated (*).

CFU-E/2×10^5 Input Cells
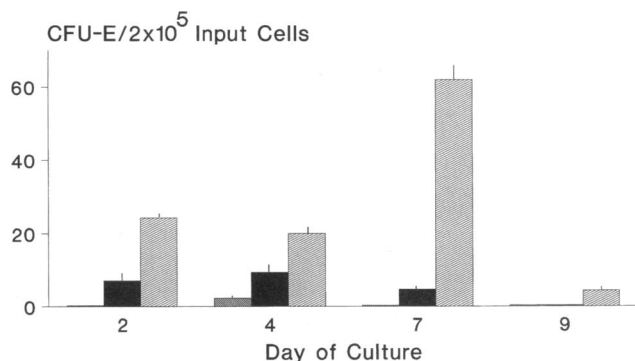
Figure 4. TPO enhances the generation of CFU-E in suspension culture. A two-stage culture system was used to assess the influence of rmTPO on the generation of late erythroid progenitors. Nonadherent murine marrow cells were cultured with either 2,000 U/ml rmTPO ([■]), 2 U/ml recombinant human Epo plus sham medium (■), or Epo plus TPO ([▨]). On days 2, 4, 7, and 9 of culture aliquots of cells were removed and CFU-E quantitated by standard methods (27). The results represent the mean number CFU-E±SEM of three assays per data point from a single experiment. Similar results were obtained in three additional experiments, one of which used purified rmTPO.

argue that a major component of the protective effects of TPO on erythrocyte levels after cytoreductive therapy was enhanced red cell production.

To determine the level at which TPO influenced erythropoiesis, we studied its effects on the production of erythroid progenitors in vitro. In our initial studies of its biological activity, we tested rmTPO, alone and together with Epo and PWM-SCM for enhancement of BFU-E and CFU-E development (5). Those studies failed to reveal any statistically significant influence on in vitro erythropoiesis. However, the studies did not use purified cytokines (PWM-SCM contains multiple cytokines including TPO) and did use only a single, high concentration of Epo. To further address this question, we performed in vitro assays for BFU-E and CFU-E and for the generation of the late erythroid progenitors from normal marrow cells using multiple concentrations of purified cytokines with and without TPO. In the presence of high levels of purified IL-3 or KL and Epo, both the number and size of BFU-E–derived colonies increased if TPO was also present (Fig. 3). In addition, when marrow cells were placed in suspension culture in the presence of TPO plus Epo, the number of output CFU-E was substantially greater than in the presence of Epo alone (Fig. 4). Together, these findings suggest an explanation for the effects of TPO on erythropoiesis in vivo. Previous studies have demonstrated that mice that have undergone myelosuppressive therapy have high circulating levels of both IL-3 and Epo (31, 32). Therefore, our administration of exogenous TPO to such animals would be expected to approximate the in vitro conditions shown to enhance the generation of erythroid progenitors in the present study. In the presence of elevated Epo levels, the expanded pool of erythroid progenitors develop into reticulocytes and mature erythrocytes. In the absence of elevated levels of IL-3 or KL and Epo (e.g., in our experiments with normal animals, Table I), the effects of TPO on erythropoiesis are minimal.

In this report we have shown that TPO affects the generation and development of early and late erythroid progenitors, both in vitro and in vivo, and leads to enhanced erythropoietic recovery after myelosuppressive therapy. Although cytokines that act

to expand early hematopoietic progenitor cells, such as IL-3, KL, IL-6, and IL-11 display wide-ranging effects on a number of different cell lineages (10–14, 33, 34), including effects on erythropoietic progenitors in vivo (35–40), late-acting cytokines, including Epo, G-CSF, M-CSF, IL-5, and TPO were initially thought to be lineage-specific (7, 8, 15, 16, 41, 42). However, more recent findings suggest that many of these cytokines may also affect other cell lineages. For example, G-CSF has been shown to act in synergy with IL-3 or KL to trigger very early hematopoietic progenitors into the cell cycle, and M-CSF affects placental development and osteoid cells (43–45). Of considerable interest for the present studies, Epo has been shown to augment megakaryocyte formation in the presence of aplastic serum, a known source of TPO (23, 24), and to expand not only erythroid but also megakaryocyte and granulocytic progenitors in Epo-treated patients (46). Therefore, our results have both physiologic and therapeutic implications. First, recognition of the interaction between TPO and erythropoiesis adds to our growing appreciation of the considerable pleiotropy exhibited by hematopoietic cytokines (47). Second, as TPO influences multiple hematopoietic progenitors in the proper environment, elevated levels of the cytokine can impact the development of other blood cell types. These findings suggest that the therapeutic use of TPO in iatrogenic and natural states of marrow failure may be greater than initially anticipated.

Acknowledgments

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