Thrombopoietin: More than a Lineage-Specific Megakaryocyte Growth Factor

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ABSTRACT

In the past two years thrombopoietin (TPO) has been cloned, its effects on cells of the megakaryocytic lineage have been described in detail and its use in clinical settings of myelosuppressive therapy has begun. Moreover, the mechanisms by which the hormone binds to its receptor have been studied in detail, and the intracellular pathways employed during TPO signaling have been extensively explored. Although most workers in the field predicted that TPO would be lineage-specific, with physiologic effects limited to megakaryocytes and platelets, several features of Mpl biology suggest its influence on hematopoiesis may be more widespread than initially anticipated. To test this possibility, we and others have begun to explore whether TPO affects development of the hematopoietic stem cell. In suspension culture, TPO alone can support the survival of a fraction of hematopoietic stem cells but does not lead to their proliferation. However, in combination with interleukin 3 or stem cell factor, TPO accelerates hematopoietic stem cell entry into the cell cycle over that seen with these early-acting cytokines alone, increases the number of subsequent cell divisions per unit time and results in the output of far greater numbers of colony-forming cells of all lineages. Conclusions from these in vitro studies are supported by two types of in vivo experiments. The administration of TPO to either normal or myelosuppressed animals causes an expansion of colony-forming unit (CFU)-megakaryocyte, CFU-granulocyte-macrophage, CFU-granulocyte/erythroid/macrophage/megakaryocyte and BFU-E, and elimination of TPO or its receptor by genetic engineering results in a substantial decrease in the numbers of these progenitors in both the marrow and spleen. It is thus becoming clear that the effects of TPO extend beyond that of a megakaryocyte-specific factor and suggest that the hematopoietic effects of administration of the hormone may be greater than initially anticipated. *Stem Cells 1997;15(suppl 1):97-103*

INTRODUCTION

Although initially proposed nearly 40 years ago, the existence of a specific regulator of megakaryocyte and platelet production was not assured until thrombopoietin (TPO) was cloned in 1994 [1-5]. Over the past three years, a number of investigators have reported that the recombinant protein fulfills most of the criteria proposed for the primary regulator of the megakaryocytic lineage [6, 7]. These properties include a profound effect on megakaryocyte proliferation and differentiation, expansion of megakaryocytic progenitor cells, an inverse relationship between platelet levels and plasma hormone concentration, and an impressive stimulation of platelet production upon its administration to normal or myelosuppressed animals [8-13]. Moreover, genetic elimination of TPO or its receptor reduces megakaryocyte and platelet numbers to 5%-15% of normal, and reduces the size and level of maturation of the residual cells [14, 15].

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Based on parallels drawn between the effects of erythropoietin on red cell production and TPO on megakaryocyte and platelet development, it was widely assumed that TPO would display lineage-specific properties. However, since its cloning as the cellular homolog of the transforming oncogene v-*mpl*, a number of observations have suggested that the c-Mpl receptor might function in cells outside the megakaryocytic lineage. For example, we and others have shown that TPO acts in synergy with erythropoietin to enhance the development of erythroid progenitor cells in vitro [16, 17], likely contributing to the enhanced erythropoiesis noted when TPO is administered to myelosuppressed animals [16]. Moreover, v-*mpl* produces a panmyeloid expansion involving erythroid, myeloid and megakaryocytic progenitors in mice infected with the MPL virus [18]. And the normal c-*mpl* cellular proto-oncogene is expressed in CD34-selected cells [19], leaving open the possibility that TPO might exert an influence on cells more primitive than megakaryocytes or the megakaryocytic colony-forming cell. Based on these observations, we have begun to explore whether the hormone affects the survival or proliferation of the hematopoietic stem cell.

Thrombopoietin Promotes the Survival of Hematopoietic Stem Cells

A number of investigators have established methods to purify cellular populations highly enriched in hematopoietic stem cells. These methods have relied heavily upon both positive and negative selection with monoclonal antibodies. Previous studies in Seattle have shown that such a highly enriched stem cell population can be obtained using a cocktail of seven monoclonal antibodies to deplete mature, lineage-committed cells, forward and side light scatter parameters to select a "blast window," and gating for the lowest retention of rhodamine 123 and Hoechst 33342 ($lin^-/Ho^{lo}/Rh^{lo}$ cells [20]). Approximately 1 in 3×10^5 marrow cells displays these properties; the population contains virtually no colony-forming cells and at most 1% day 12 colony-forming unit-spleen (CFU-S). Ten such cells reliably repopulate all of hematopoiesis when injected into lethally irradiated mice, arguing that these sorting parameters produce a homogenous population of long-term repopulating hematopoietic stem cells ([20] and *Eva Sitnicka*, personal communication). To determine if TPO could affect such cells, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) on 20 such cells and could easily detect c-*mpl* transcripts (Fig. 1). Previous investigators have shown that human CD34-selected cells, a larger population of which the $lin^-/Ho^{lo}/Rh^{lo}$ cells are also a part, display c-*mpl* [19]. Next, in order to functionally assess the contribution of c-*mpl* to stem cell biology, we determined whether TPO could support the survival of such cells. Individual $lin^-/Ho^{lo}/Rh^{lo}$ cells were sorted into 96-well plates and cultured in the presence of control medium, recombinant murine (rm) TPO, interleukin 3 (IL-3) or stem cell factor (SCF). Cultures were examined daily and the cell number recorded. As shown in Figure 2, three-quarters of the cells died within two days in medium alone, and all were dead by day 4; in contrast, IL-3 or SCF alone was able to support the survival of the majority of cells for at least one week. The delayed addition of the combination of IL-3, SCF and IL-6 for up to one week enabled surviving cells to display their full developmental potential. Although less efficient, TPO was also able to support the survival of a proportion of the $lin^-/Ho^{lo}/Rh^{lo}$ cells. Moreover, the delayed addition of IL-3, SCF and IL-6 supported vigorous proliferation of single surviving cells, which developed into colony-forming cells of all hematopoietic lineages when subsequently plated in semisolid clonal assays.

TPO Acts in Synergy with IL-3 or SCF to Promote the Proliferation and Differentiation of Hematopoietic Stem Cells.

Few of the cells which survived in the presence of TPO alone in the experiment illustrated in Figure 2 divided even once; most persisted for the duration of the experiment as single cells. In contrast, in the presence of either IL-3 or SCF, the addition of TPO induced multiple effects on cellular proliferation over that seen with IL-3 or SCF alone. For example, 32% of cultured single $lin^-/Ho^{lo}/Rh^{lo}$ cells underwent either one or two divisions in two weeks in the presence of SCF alone. When TPO was added, 91% of the clones divided up to seven times. The single cell cloning efficiency increased from 27% in IL-3 alone to 57% in IL-3 plus TPO, although unlike the results with SCF, the already substantial degree of

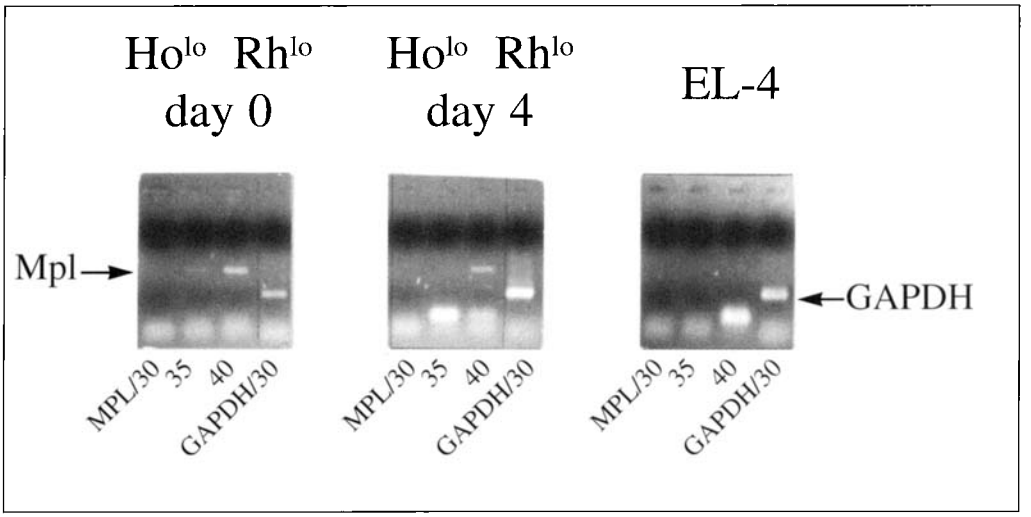

Figure 1. RT-PCR analysis of highly purified primitive hematopoietic cells. *Lin$^-$/Ho^{lo}/Rh^{lo} cells were purified and RNA extracted and reverse transcribed either before (day 0) or after (day 4) culture in IL-3/SCF/IL-6. Aliquots of 20 cells were subjected to 30, 35 or 40 cycles of PCR using primers that flank introns 10 and 11 and that should produce a 450 bp fragment if derived from mRNA (>3 kb if from genomic DNA). Reverse transcribed RNA from EL-4 cells served as a negative PCR control, and glyceraldehyde-3-phosphate dehydrogenase primers serve as a control for RNA integrity.*

clonal expansion induced by IL-3 was not appreciably increased by adding TPO. Despite this minor caveat, however, the addition of TPO induced proliferation in single cells which would otherwise have remained dormant in the presence of IL-3 or SCF alone.

In addition to these effects on cloning efficiency, TPO also induced IL-3 or SCF-treated cells to enter the cell cycle earlier than in the presence of these early-acting cytokines alone [21]. For example, by day 2 of culture, 12% of the cells that would ultimately divide in the presence of SCF alone had done so; adding TPO to the culture increased this to 20%. For IL-3, by day 3 only 9% of the cells that ultimately would divide had already undergone one division, in contrast, 23% had divided at least once by day 3 in the presence of IL-3 plus TPO. The results of these two findings, increased cloning efficiency and acceleration of cell division in the presence of TPO, are shown in Figure 3; the total number of stem

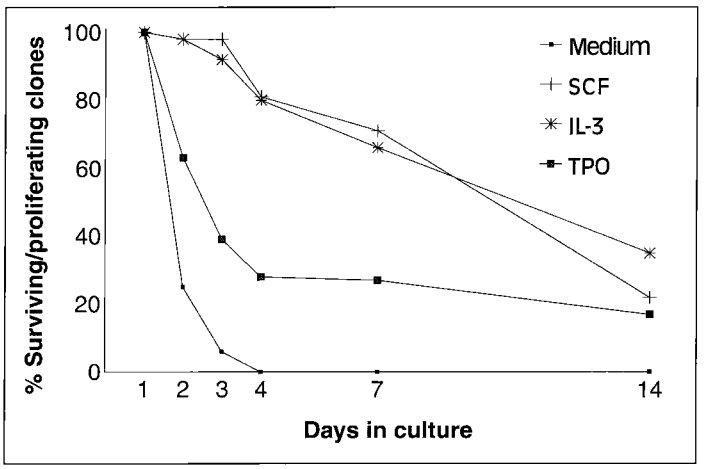

Figure 2. TPO supports the survival of primitive hematopoietic cells. *Single lin$^-$/Ho^{lo}/Rh^{lo} cells were sorted into wells of a 96-well plate and cultured for 14 days in control medium, 20 ng/ml rmSCF, 100 ng/ml rmIL-3 or 5 ng/ml rmTPO. Cell numbers per well were assessed daily. The percentage of wells which shows either single (survival) or multiple (proliferating) cells is shown.*

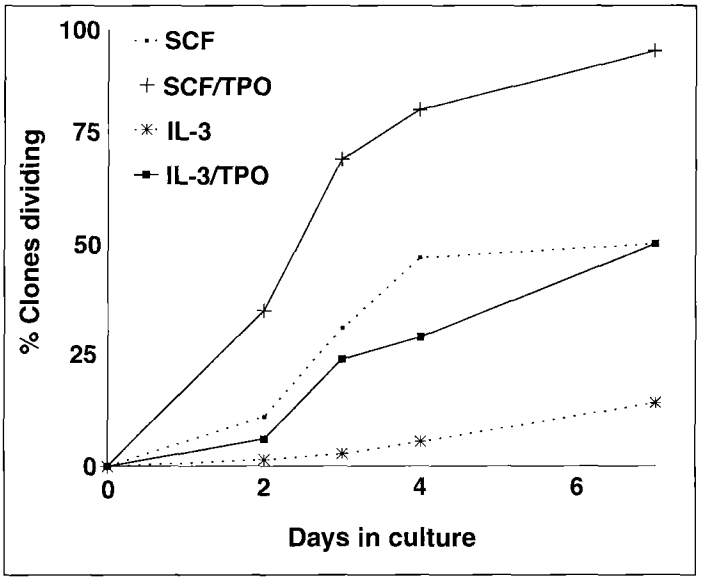

Figure 3. TPO enhances the proliferation of primitive hematopoietic cells in the presence of either IL-3 or SCF. *Single-cell cultures were initiated with either IL-3 or SCF with or without TPO, and the percentage of wells containing greater than a single cell was scored daily.*

cells which begin to proliferate in the presence of either IL-3 or SCF plus TPO is far greater than that seen with either early-acting cytokine alone.

Similar results were recently reported by *Ogawa* and coworkers. Using highly purified, cell cycle dormant, primitive progenitors from 5-fluorouracil-treated mice, these investigators showed that TPO acted in synergy with either IL-3 or SCF to augment the numbers of multiple types of hematopoietic colonies which develop in semisolid cultures, including those derived from granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) [22]. Single-cell mapping experiments revealed that TPO acted on these multilineage progenitors to speed their entry into the cell cycle. Moreover, in suspension culture, the addition of TPO to SCF lead to far greater expansion of total cell numbers and of CFU-GM (granulocyte-macrophage) and CFU-GEMM progenitors than seen with SCF alone. More recently, *Ogawa* has extended these conclusions to primitive human hematopoietic cells. Using $CD34^{+}/CD38^{lo/-}$/c-kit^{lo} cells, these investigators reported that the addition of TPO to either IL-3 or SCF lead to the enhanced production of hematopoietic colonies of multiple lineages in semisolid culture, and to greater expansion of the numbers of these same progenitor cell types in suspension culture [23]. Furthermore, the synergistic effects of TPO were more pronounced than those seen with IL-6, IL-11 or G-CSF. Thus, TPO exerts a direct (i.e., at the single-cell level) but dependent (on IL-3 or SCF) proliferative effect on primitive hematopoietic cells including the stem cell.

The In Vitro Multilineage Effects of TPO Correlate with Its in Vivo Biology

Two lines of evidence suggest that the effects of TPO revealed by in vitro studies with primitive hematopoietic cells correlate well with its properties in vivo. First, administration of exogenous TPO to normal mice increases the total number of BFU-E, CFU-GM and CFU-MK in marrow and spleen [24]. Its administration to myelosuppressed mice also accelerates the recovery of these same progenitor cell types and of CFU-E (erythroid), leading to enhanced production not only of platelets, but also of neutrophils and reticulocytes [16, 25]. Similar effects on the recovery of all types of hematopoietic progenitor cells in monkeys treated with radiation have also been presented [26]. Second, genetic elimination of TPO or its receptor is associated with a substantial decrease in the numbers of all types of hematopoietic progenitors, including the primitive multilineage CFU-blast [15, 27]. Thus, both gain-of-function and loss-of-function in vivo TPO experiments are consistent with its in vitro activities on primitive hematopoietic cells.

Conclusions

Although initially thought to be lineage-specific, TPO displays prominent direct effects on primitive hematopoietic cells of multiple lineages. In vitro, TPO can support the survival of a proportion of hematopoietic stem cells, and in synergy with either IL-3 or SCF can enhance their proliferative responses. Cells not stimulated to divide by either cytokine alone begin to proliferate if TPO is also present, and their entry into the cell cycle is accelerated. Suspension culture expansion studies indicate that TPO augments the number of hematopoietic progenitors of all types derived from SCF-stimulated, highly purified, cell cycle dormant, primitive hematopoietic progenitors over that seen with cells stimulated with SCF alone. In vivo, the effects of TPO excess or deprivation on primitive stages of hematopoiesis are also clear. The administration of TPO to normal or myelosuppressed animals results in enhanced numbers of both multilineage and unipotent hematopoietic progenitors of all types, and elimination of TPO function by genetic engineering of either the hormone or its receptor substantially reduces hematopoietic progenitor numbers of all classes and levels of maturity. Together, these results strongly suggest that TPO plays a more widespread role in supporting hematopoiesis than previously recognized and imply that the hematologic response to its therapeutic administration may be greater than initially anticipated.

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Discussion

Dr. Torok-Storb: You showed the effect of a single dose of thrombopoietin on day 1 or 3 post-myeloablation. You saw cells coming out faster than after a single dose given on day 3.

Dr. Kaushansky: With a single dose on day 1, recovery was accelerated. With a single dose on day 3, recovery occurred on the same day as control, only the platelet count overshot normal valves.

Dr. Torok-Storb: And the one-day dose stayed up?

Dr. Kaushansky: There was a slight overshoot with day 1 TPO, but the platelet count rapidly returned to baseline levels. There wasn't the overshoot that you saw with the day-3 dosage.

Dr. Torok-Storb: So how do you interpret the overshoot?

Dr. Kaushansky: I think that giving it later, day 3, day 5, day 7, or continuously, there is an effect on progenitor cells and on mature cells which accounts for the overshoot. But, I think that the critical effect in promoting earlier recovery of all the lineages is this very early effect that occurs within the first one or two days. I would argue that this is probably an effect working on the stem cell, or its immediate progeny. We are now starting experiments to test that hypothesis.

Dr. Fibbe: You are probably aware of studies in primates that highly purified stem cell grafts are given and TPO was applied. In these experiments, TPO had almost no effect on platelet recovery. The most likely explanation for that, of course, is that in the setting of minimal numbers of stem cells, TPO has no effect and that the presence of more mature progenitors would be important.

Dr. Kaushansky: I cannot reconcile those observations. This is not a transplant model with myeloablation; this is myelosuppression. These animals all recover within three or four weeks. Thus, there are reasonable numbers of progenitor cells in our animals.

Dr. Broxmeyer: I would like to add something to some of the data you showed. If you add TPO to TPO knockouts, you get the progenitor numbers back.

Dr. Moore: I have some data that support some of your points using an adenovirus vector to produce thrombopoietin. If we administer the adenoviral TPO interperitoneally with radiation and carboplatin treatment as you showed with your administration of systemic thrombopoietin, it not only accelerates platelet recovery, but also granulocyte and hematopoietic stem cell recovery. When we give that same vector to an untreated immunocompromised SCID mouse who lacks an immune response to the adenovirus, we get platelet counts of 15 to 18 million per mm^3 for about three months, and we get granulocyte levels of 80,000 to 100,000/mm^3 in some mice maintained for some weeks. However, the hematocrit falls. Obviously, the animals cannot sustain that tremendous overproduction of platelets and granulocytes, but there is competition and they become anemic. I think this is a space phenomenon because the spleen and the marrow are just filled with megakaryocytes and granulocytes. The final thing is, if we do that same adenovector TPO in a normal immunocompetent BALB/c mouse, they get a transient high platelet count, but they may become thrombocytopenic because they develop antibodies to their own thrombopoietin, universally, actually. Then they become leukopenic, and when we measure the progenitors they are all reduced in the marrow by 50% to 75%, i.e., CFU-GEMM, BFU-E. So it is like your mpl-mouse.

Dr. Hoffman: One of the things we became acutely aware of when we purified human stem cells is that our populations of primitive progenitor cells co-purified with human hematopoietic stem cells. So were those rhodamine low populations assayed for more primitive megakaryocytic progenitor cells?

Dr. Kaushansky: We have not had the same success that you have in growing BFU-Meg versus CFU-Meg. I can only tell you that when we set up our standard CFU-Meg assay, there are many colonies in the right conditions that resemble what you have described as BFU-Meg. So although we are looking at seven days, I believe that our assay is picking up a similar cell. We have never seen any megakaryocyte colonies, either small and unifocal or large and multifocal, when we plated cells from our stem cell population grown for zero to three days in suspension culture. Thus, our population is devoid of colony-forming cells.

Dr. Hoffman: Does TPO have a proliferative effect on single cells of the stem cell type, or does it prevent apoptosis?

Dr. Kaushansky: Even going out to a week or 10 days, about a third of the stem cells are still in the wells. Most of them still are just single cells. They are not two or four or eight. So I would argue the effect is on survival. However, it would be tough to look for apoptosis on single cells. In contrast, the proliferative effects were primarily in combination with c-*kit* ligand or with IL-3.

Dr. Ihle: Just a precautionary note regarding the kinds of complications that can exist when you look in vivo for physiological loops. And this came out of some very recent knockout experiments that we are trying to understand. For example, a lot of cytokines, including thrombopoietin, cause the activation of Stat5. One of the genes regulated by Stat5 is oncostatin M. Oncostatin M is able to induce G-CSF production by cells. So immediately if you start stimulating cells in vivo with TPO, oncostatin M is produced and it is going to have its physiological effect as well as causing the production of new cytokines. I think that these studies can be very complex.