Thrombopoietin expands hematopoietic stem cells after transplantation

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Multiple lines of evidence indicate that thrombopoietin (TPO) contributes to the development of hematopoietic stem cells (HSC), supporting their survival and proliferation in vitro. To determine whether TPO supports the impressive expansion of HSC observed following transplantation, we transplanted normal marrow cells into lethally irradiated Tpo−/− and Tpo+/+ mice and quantified HSC self-renewal and expansion and hematopoietic progenitor cell homing. Although essentially identical numbers of marrow-associated colony forming unit–culture (a surrogate measure of stem cell homing) were observed in each type of recipient 24 hours following transplantation, we found that a minimum of fourfold greater numbers of marrow cells were required to radioprotect Tpo-null mice than to radioprotect controls. To assess whether long-term repopulating (LTR) HSCs self-renew and expand in Tpo−/− recipients or controls, we performed limiting-dilution secondary transplants using donor cells from the Tpo−/− or Tpo+/+ recipients 5–7.5 weeks following primary transplantation. We found that LTR HSCs expand to levels 10–20 times greater within this time period in normal recipients than in Tpo-null mice and that physiologically relevant amounts of TPO administered to the Tpo−/− recipients could substantially correct this defect. Our results establish that TPO greatly promotes the self-renewal and expansion of HSCs in vivo following marrow transplantation.


Introduction

Thrombopoietin (TPO) was first cloned and its recombinant protein first produced and characterized in 1994 (1–4). Initial studies with the recombinant protein were directed toward demonstrating that the cytokine was the primary regulator of megakaryopoiesis and platelet production. In this regard it was rapidly appreciated that the cloned gene fulfilled all of the criteria historically set out for it (5, 6). For example, TPO affects nearly every stage of megakaryocyte (MK) development from committed progenitor to the mature platelet. The hormone is the most potent cytokine to stimulate the development of MK colonies from marrow progenitors in semisolid cultures (7), and it induces the expression of glycoprotein Ib/IIa, the platelet fibrinogen receptor, and the glycoprotein Ib/IX complex, the platelet vWF receptor, in immature MKs (5). In the presence of the cytokine, MKs grow in size, develop several types of specific granules, and form demarcation membranes, a precursor of platelet formation. And although TPO is not essential for the last stages of platelet formation (8), it does prime platelets to respond to subthreshold levels of classic agonists such as ADP, collagen, or thrombin (9, 10).

Based on results using partially purified preparations of the hormone, previous reports concluded that TPO is a specific growth factor for the MK and platelet lineage (11, 12). However, several lines of evidence using recombinant protein now indicate that the hormone exerts a profound influence on hematopoietic stem cells (HSCs). Using highly purified populations of marrow cells, two groups reported that TPO enhances the survival of murine HSCs in vitro and, in the presence of additional cytokines, could augment their proliferation (13, 14); this conclusion was quickly expanded to human cells (15). One of the first indications that these effects extended to in vivo hematopoiesis came with the discovery that TPO accelerates the recovery of all hematopoietic lineages following myelosuppressive therapies (16, 17). Moreover, mice genetically deficient in the TPO receptor, c-Mpl, display reduced levels of all types of hematopoietic progenitors (18), again suggesting the panhematopoietic effect of the cytokine. The profound effect of TPO on HSCs was then established when it was shown that all of the long-term repopulating (LTR) HSCs display c-Mpl, and that c-mpl−/− mice display substantial reductions in the numbers of LTR HSCs (19, 20). This physiology was...
very recently extended to humans when it was discovered that many children with congenital amegakaryocytic thrombocytopenia, the majority of whom develop aplastic anemia within a few years of birth, display many different missense and nonsense mutations in the c-Mpl receptor (21).

Although experimental hematologists have tested several approaches to expand adult HSCs in vitro, there is little reproducible evidence that such strategies have been successful. In contrast, if a limited number of stem cells are transplanted into a lethally irradiated animal, within a few months the number of stem cells in the marrow and spleen of the recipient is in great excess of input numbers. For example, in one report of serial transplantation experiments, about ten times more LTR HSCs were found with each successive transfer of cells (22). Given the accumulating data indicating a role for TPO in stem cell biology, in the present work we sought to test whether the hormone is important for the self-renewal and expansion of HSCs that follow marrow transplantation. We report that, for both short-term radioprotection and long-term repopulation, TPO is vital, enhancing survival following lethal irradiation at least fivefold compared with that seen in the absence of the hormone, and accelerating the renewal and repopulation of the marrow HSC pool 13- to 20-fold in the first 2 months following transplantation.

**Methods**

**Mice and animal care.** All mice used in this study were C57BL/6. The initial stem cell donors were CD45.1 and were obtained from Taconic (Germantown, New York, USA); all others were of the CD45.2 genotype (The Jackson Laboratory, Bar Harbor, Maine, USA). TPO-null mice bred onto the C57BL/6 background were kindly provided by Frederic de Sauvage (Genentech Inc., South San Francisco, California, USA) and have been previously described (23). Mice were housed in a specific pathogen–free environment, and the Animal Care Committee of the University of Washington approved all protocols involving mice.

**Reagents.** Recombinant murine TPO was kindly provided by Akihiro Shimosaka (Kirin Pharmaceuticals Inc., Gunma, Japan). It was administered to some recipient animals by subcutaneous injection once every 3 months following the procedure and analyzed by flow cytometry using a phycoerythrin-labeled mAb to murine c-Mpl receptor (21).

**Marrow cell transplantation.** Marrow cell transplantation was performed using standard protocols; mice were subjected to 1150 cGy whole-body irradiation from a 137Cs source, and then infused with donor cells by tail vein injection. Mice were fed standard mouse chow, and antibiotics were administered in the drinking water for 1 week prior to and 3 weeks following transplantation. For secondary transplants, 5–7.5 weeks following primary marrow cell infusion, mice were sacrificed; then single-cell suspensions of femoral, tibial, and pelvic marrow were prepared and infused into normal CD45.2 recipients and compared post-transplant survival and percent CD45.1 positivity were determined at the indicated number of weeks. ND, not determined; +TPO, mice were administered 80 ng recombinant TPO twice to thrice weekly until sacrifice.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Radioprotection and short-term repopulation in Tpo−/− mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipient</strong></td>
<td><strong>Donor cell no.</strong></td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Tpo−/−</td>
<td>2 × 10^6</td>
</tr>
<tr>
<td>Tpo−/−</td>
<td>2 × 10^6</td>
</tr>
<tr>
<td>Tpo−/−</td>
<td>1 × 10^6</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>Tpo−/−</td>
<td>1 × 10^6</td>
</tr>
<tr>
<td>Tpo−/−</td>
<td>1 × 10^6</td>
</tr>
<tr>
<td>Tpo−/− (+TPO)</td>
<td>1 × 10^6</td>
</tr>
</tbody>
</table>

Normal or Tpo−/− recipients were infused with the indicated number of CD45.1+ marrow cells, and survival and percent CD45.1+ positivity were determined at the indicated number of weeks. ND, not determined; +TPO, mice were administered 80 ng recombinant TPO twice to thrice weekly until sacrifice.

**Results**

TPO enhances the capacity of transplanted marrow cells to radioprotect. In order to determine whether TPO was important for the ability of short-term repopulating stem cells to radioprotect recipient mice, we transplanted 2 × 10^6 normal CD45.1 marrow cells into normal CD45.2 recipients and compared post-transplant survival to that of CD45.2 Tpo−/− mice transplanted with either 2 × 10^6 or 1 × 10^6 normal CD45.1 marrow cells. As shown in Table 1, we found that nine of ten
normal recipients of $2 \times 10^4$ normal marrow cells survived; a mean of 86% of the hematopoietic cells were CD45.1$^+$ in the recipients. In contrast, only two of ten $Tpo^{+/−}$ recipients of the same number of normal marrow cells survived, with 80% of those hematopoietic cells CD45.1$^+$. The infusion of $1 \times 10^6$ normal marrow cells was required to rescue eight of ten transplanted $Tpo^{+/−}$ mice. In a second experiment, $1 \times 10^6$ normal marrow cells were injected into four $Tpo^{+/+}$ recipients, four $Tpo^{+/−}$ recipients that were given saline thrice weekly, and four $Tpo^{−/−}$ recipients given thrice-weekly injections of 80 ng recombinant murine TPO. This dose and dosing schedule of TPO was determined to be the most physiologically relevant, as it returned the platelet count of $Tpo^{+/−}$ mice to normal levels and maintained it at those levels. All four of the $Tpo^{+/−}$ recipients survived more than 5 weeks; in contrast, only one of four of both $Tpo^{+/+}$-recipient groups survived more than 5 weeks. Thus, since we saw little or no radiation-induced death in the control groups, the four- to fivefold enhanced radioprotection afforded by normal marrow cells in lethally irradiated normal mice compared with $Tpo^{−/−}$ mice is a minimal estimation.

**TPO does not affect the homing of hematopoietic cells to marrow or spleen.** The previous results assessing radioprotection and short-term repopulation could have been due either to reduced stem cell homing to marrow in $Tpo^{+/−}$ mice or to defective expansion of the stem cells lodged in the $Tpo^{+/−}$ marrow. To distinguish between these possibilities, in two sets of experiments we transplanted normal marrow containing either $1.8 \times 10^5$ or $6.3 \times 10^5$ CFU-C into groups of three to five lethally irradiated $Tpo^{+/−}$ or normal mice and quantitated CFU-C recovered in the marrow 24 hours later. Previous studies have indicated that CFU-C homing is an accurate surrogate for stem cell homing (24–26). As shown in Table 2, we found that in both sets of experiments cells homed as efficiently in $Tpo^{+/−}$ recipients as in wild-type mice, the values in $Tpo^{+/−}$ mice being 89% and 112% of normal for homing to the marrow, and 163% and 94% of normal for lodgment in the spleen, respectively. In a second type of analysis, we labeled normal donor marrow cells with the membrane-soluble dye CFSE and assessed the percentage of marrow cells that were CFSE$^+$ 24 hours following their infusion into lethally irradiated recipients. We found a mean of 1.4% CFSE$^+$ marrow cells in $Tpo^{+/−}$ recipients and 1.3% CFSE$^+$ in the $Tpo^{−/−}$ recipients. Thus, the difference in radioprotection and short-term repopulation following transplantation of normal marrow cells into $Tpo^{+/−}$ and $Tpo^{−/−}$ mice was not due to abnormal hematopoietic cell homing in the latter group of mice.

TPO enhances the self-renewal and expansion of LTR HSCs. Previous studies demonstrated that upon infusion into lethally irradiated mice, HSCs expand within the first few months following the procedure. To determine whether TPO affects this aspect of stem cell physiology and to quantitate the effect, limiting-dilution secondary transplants were performed using marrow cells from the $Tpo^{+/+}$ or $Tpo^{−/−}$ primary transplant recipients from the two experiments reported upon in Table 1, 5 or 7.5 weeks following primary transplantation. In the first experiment, the $Tpo^{+/+}$ and $Tpo^{−/−}$ mice transplanted with $2 \times 10^5$ normal marrow cells 7.5 weeks earlier were sacrificed, and $3 \times 10^5$ marrow cells from the normal primary transplant recipients and either $1 \times 10^6$ or $2.5 \times 10^5$ marrow cells from the $Tpo^{−/−}$-primary transplant recipients were infused into normal, lethally irradiated C57BL/6 secondary recipients. In the second experiment, the primary transplant recipients were sacrificed at 5 weeks, and limiting-dilution secondary transplants were performed to calculate HSC expansion during the primary transplant. In the second experiment we also studied whether the administration of TPO during the primary transplant period could correct for the genetic deficiency. The survival curves from the first set of secondary transplants are shown in Figure 1, and the results from both

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**Table 2**

Marrow and splenic homing of normal marrow cells in $Tpo^{−/−}$ mice

<table>
<thead>
<tr>
<th>Recipient</th>
<th>No. of CFU-C infused</th>
<th>$n$</th>
<th>% Marrow recovery</th>
<th>% Splenic recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1, day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Tpo^{+/−}$</td>
<td>$1.8 \times 10^5$</td>
<td>5</td>
<td>10.9 ± 0.25</td>
<td>3.0 ± 0.17</td>
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<tr>
<td>$Tpo^{−/−}$</td>
<td>$1.8 \times 10^5$</td>
<td>5</td>
<td>9.7 ± 0.60</td>
<td>4.9 ± 0.86</td>
</tr>
<tr>
<td>Experiment 2, day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Tpo^{+/−}$</td>
<td>$6.3 \times 10^4$</td>
<td>3</td>
<td>17 ± 4.2</td>
<td>5.3 ± 1.3</td>
</tr>
<tr>
<td>$Tpo^{−/−}$</td>
<td>$6.3 \times 10^4$</td>
<td>3</td>
<td>19 ± 2.3</td>
<td>4.9 ± 0.60</td>
</tr>
</tbody>
</table>

Marrow cells containing a known number of CFU-C were infused into lethally irradiated $Tpo^{+/+}$ or $Tpo^{−/−}$ recipients, and the mice were sacrificed 24 hours later. The results represent the mean number of CFU-C (± SEM) recovered 24 hours following their infusion into lethally irradiated recipients.

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**Figure 1**

Survival of mice transplanted with marrow cells from previously transplanted $Tpo^{+/+}$ or $Tpo^{−/−}$ mice. The curves shown are survival as a function of time for: normal recipient mice transplanted with $3 \times 10^5$ marrow cells from normal recipient mice transplanted 7.5 weeks earlier with $2 \times 10^5$ normal marrow cells (solid line); $1 \times 10^6$ marrow cells from $Tpo^{−/−}$ mice transplanted 7.5 weeks earlier with $2 \times 10^5$ normal marrow cells (dashed line); and $2.5 \times 10^5$ marrow cells from $Tpo^{−/−}$ mice transplanted 7.5 weeks earlier with $2 \times 10^5$ normal marrow cells (dotted line).
Table 3
Secondary transplantation from Tpo+/+ and Tpo–/– primary transplant recipients

<table>
<thead>
<tr>
<th>Donor</th>
<th>(No. of cells in 1°)</th>
<th>Time of 2° (wks)</th>
<th>No. of cells in 2°</th>
<th>No. of recipients</th>
<th>% Survival</th>
<th>Relative expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tpo+/+</td>
<td>(2 × 10^6)</td>
<td>7.5</td>
<td>3 × 10^6</td>
<td>10</td>
<td>40</td>
<td>(1.0)</td>
</tr>
<tr>
<td>Tpo–/–</td>
<td>(2 × 10^6)</td>
<td>7.5</td>
<td>1 × 10^6</td>
<td>10</td>
<td>10</td>
<td>0.076</td>
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<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tpo+/+</td>
<td>(1 × 10^6)</td>
<td>5</td>
<td>5 × 10^6</td>
<td>10</td>
<td>100</td>
<td>(1.0)</td>
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<tr>
<td>Tpo–/–</td>
<td>(1 × 10^6)</td>
<td>5</td>
<td>7.5 × 10^6</td>
<td>10</td>
<td>70</td>
<td>0.048</td>
</tr>
<tr>
<td>Tpo–/– + TPO</td>
<td>(1 × 10^6)</td>
<td>5</td>
<td>1 × 10^6</td>
<td>10</td>
<td>80</td>
<td>0.40</td>
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<tr>
<td>Tpo–/– + TPO</td>
<td>(1 × 10^6)</td>
<td>5</td>
<td>7.5 × 10^6</td>
<td>10</td>
<td>100</td>
<td>NC</td>
</tr>
</tbody>
</table>

Mice having received primary transplants 5–7.5 weeks earlier were sacrificed, and limiting dilutions of marrow cells were infused into C57BL/6 recipients and their survival monitored. The inocula were composed of marrow cells mixed from multiple donor mice, except in the Tpo–/– and Tpo–/– + TPO groups in experiment 2, in which only a single primary transplant recipient of each group had survived to serve as a secondary transplant donor. At 5 months, all of the surviving mice displayed a majority of CD45.1+ hematopoietic cells in which only a single primary transplant recipient of each group had survived to serve as a secondary transplant recipient. The relative expansion of HSCs in the 5–7.5 weeks in the primary transplant recipients was calculated based on the number of cells infused into the secondary recipients and the relative survival of the secondary transplant recipients. The calculation of relative expansion in the Tpo–/– and Tpo–/– + TPO low-cell dose groups in experiment 2 is a minimal estimate, as there were no deaths in the control Tpo–/– group. NC, non-calculable, since there were no deaths in either the Tpo–/– or the high-cell dose Tpo–/– + TPO groups. 1°, primary; 2°, secondary.

experiments are tabulated and the relative stem cell expansion calculated in Table 3.

If one assumes that the successful re-engraftment of, for example, four of ten mice represents the presence of twice as many HSCs in the transplant inoculum as if two of ten mice were engrafted, then one can calculate the relative numbers of stem cells present in the primary transplant recipients. As illustrated in Table 3, the number of HSCs present in the marrow of mice transplanted 5 weeks earlier with normal HSCs was approximately 20-fold greater when the transplantation inoculum was derived from transplanted normal mice than when the transplanted cells had come from Tpo–/– mice transplanted with normal HSCs. At 7.5 weeks, there were 13- to 16-fold more HSCs in the primary hematopoietic organs of mice 24 hours later. We next studied whether TPO affected expansion of LTR HSCs by performing limiting-dilution secondary transplant experiments, using our primary Tpo+/+ and Tpo–/– recipients as donors. We found a profound reduction in the capacity of HSCs to expand in Tpo–/– mice.
recipients, from a minimum of 13-fold to a maximum of 21-fold less expansion than in the wild-type mice. Although in one experiment 100% survival in the Tpo−/− control group made it impossible to gauge whether the difference in relative HSC expansion might have been even greater if we had used a smaller inoculum of cells, we doubt that this is the case, as the two experiments yielded quite similar levels of LTR HSC expansion 5 and 7.5 weeks following primary transplantation. Studies performed by Iscove and Nawa demonstrated that serial transplantation experiments of the type performed in this report lead to about tenfold increases in LTR HSC numbers over input levels (22), suggesting that TPO might account for the maximal level of stem cell expansion possible under these conditions. Moreover, experiments performed by Eaves’s group indicate that genetic alteration of the c-Kit/Steel Factor system imposes a similar level of HSC deficiency (28). In that study, transplantation of W +/+ W +/+ stem cells into normal recipients reduced HSC expansion 17-fold compared with wild-type donor cells. Although the W +/+ allele is a hypomorph and not a complete null, taken together the similar quantitative analyses suggest that TPO and Steel Factor might act synergistically on expansion of HSCs in vivo, as they do in vitro (7, 13, 14).

In the second set of our experiments we also tested whether exogenous TPO could correct the HSC expansion defect seen in Tpo-null mice. We first tested for the amount of TPO needed to provide a “replacement dose” for Tpo-null mice, as the use of pharmacological concentrations of a hormone might exert effects not seen with the physiological level. By administering different doses of recombinant TPO to Tpo-null mice, we found in preliminary experiments that 80 ng administered thrice weekly led to normalization of the platelet count after about 1 month. Using this dose of TPO in Tpo−/− recipients, we found that HSCs expand about 40% as well as in wild-type recipients. However, like the quantitative considerations raised above, this calculation is the maximal correction that could have occurred in this experiment, as all the control animals survived and repopulated. It is possible that if we had used lower numbers of marrow cells for the secondary transplants we might have seen a less impressive normalization of HSC expansion. This argument is of more than academic interest: if exogenous TPO administered to Tpo-null mice does not fully correct the stem cell defect of these animals, it is possible that the local effect of stromal cell–derived hormone is greater than that coming from the circulation. In this regard, debate remains in the field as to whether marrow stromal cell production of TPO is of importance for stress hematopoiesis.

Based on the effect of TPO on HSC self-renewal and expansion, one might predict that the administration of exogenous TPO to transplant recipients would enhance engraftment kinetics. In fact, this has been tested in both preclinical and clinical settings, and the results have been disappointing. For example, although the administration of TPO to murine stem cell transplant recipients was reported to accelerate hematological recovery in one study, the effect was quite modest (29) and did not occur in another model of marrow transplantation if TPO was given only to the recipient (30). Similarly disappointing results were reported following peripheral blood HSC transplantation into patients (31), although a modest effect was seen following the use of bone marrow stem cells (32). Moreover, we found that the administration of TPO in the short term, during the primary transplant experiments illustrated in Table 1, failed to radioprotect Tpo−/− mice given a standard number of marrow cells. These results do not necessarily cast doubt on the conclusions of the present study, as even the levels of TPO that are normally present in mice were sufficient to at least partially correct the LTR HSC expansion defect in Tpo−/− mice when administered exogenously in our experiments. Moreover, given the higher-than-normal levels of TPO that occur in animals and patients following myelosuppressive therapy, such as the conditioning therapy for transplantation, it is very likely that endogenous TPO is sufficient to provide for HSC self-renewal and expansion following transplantation. In our studies we failed to find an effect of exogenous TPO on short-term radioprotection, in contrast to the results with LTR HSCs. It is possible that the effect of TPO on stem cell expansion requires more time than is required to radioprotect a mouse, or that a minor effect could have been revealed if additional dose-finding experiments had been performed. Thus, whether exogenously administered TPO is useful for clinical transplantation will require additional study.

Finally, although we use the term “expansion” throughout this report to indicate that the numbers of HSCs found 5 or 7.5 weeks following transplantation are greater than the numbers of input cells, it is possible that TPO affects both HSC survival and proliferation, or that it only acts on the former. In this regard, using p53-null mice, Pestina and colleagues recently reported that TPO accelerates blood cell recovery following the administration of otherwise lethal chemoradiotherapy by preventing programmed cell death of hematopoietic cells (33). As recovery from such toxic insults is thought to be dependent on primitive marrow hematopoietic cells, it is possible that the effect of TPO on HSC expansion might be dependent on the same effect. The relative importance of these two fundamental processes on TPO-induced HSC expansion following transplantation deserves further study.

Acknowledgments

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