RAPID COMMUNICATION

Murine Thrombopoietin mRNA Levels Are Modulated by Platelet Count

By John M. McCarty, Katherine H. Spruget, Norma E. Fox, Daniel E. Sabath, and Kenneth Kaushansky

The activity of the c-Mpl ligand on hematopoietic progenitors meets criteria expected for thrombopoietin (TPO). Bioassays have shown that blood TPO levels are inversely related to platelet mass. We sought to identify the molecular basis for this regulation. To determine if TPO mRNA levels respond to platelet demand, RNA from selected organs of mice with high, normal, or low platelet counts was subjected to semiquantitative reverse transcriptase-polymerase chain reaction. Although no differences in TPO mRNA levels between control and treated mice could be detected in liver or kidney, TPO-specific bands were more intense after 25 to 30 polymerase chain reaction cycles in marrow-derived mRNA from thrombocytopenic mice. The TPO-specific bands were less intense in thrombocytopenic mouse marrow and spleen than control mouse marrow and spleen after 30 cycles. These data support the hypothesis that TPO levels are regulated, at least in part, by modulating mRNA levels in response to platelet demand.

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MURINE THROMBOPOIETIN mRNA REGULATION

RAMPS-treated and irradiated mice and in spleen-derived mRNA in RAMPS-treated animals. The TPO-specific bands were less intense in thrombocytotic mouse marrow and spleen than in controls. The findings that TPO mRNA levels are upregulated in marrow and spleen by thrombocytopenia and may be downregulated by TPO-induced thrombocytosis support the hypothesis that TPO levels are regulated, at least in part, by alterations in steady-state mRNA levels.

MATERIALS AND METHODS

Animal preparation. Female C57Bl/6 mice of 5 to 6 weeks of age were studied in four separate groups. All groups had baseline and posttreatment peripheral blood counts determined by Cell-Dyn 3000 counter (Abbott Laboratories, North Chicago, IL). After killing the mice, liver, spleen, both kidneys, and femurs were obtained from each mouse and flash frozen in liquid nitrogen. Experiment 1 consisted of two mice. One mouse was exposed to a single dose of 550 Gy from a cesium source (GammaCell 40 Irradiator; Atomic Energy of Canada, Ltd, Kanata, Ontario, Canada) and received 1.2 mg of carboplatinum (Bristol Meyers-Squibb Oncology Division, Princeton, NJ) intraperitoneally (IP). Another untreated mouse remained as control. Both mice were killed at day 13. Experiment 2 consisted of three mice. One mouse was exposed to a single dose of 600 Gy from a cesium source and received 1.2 mg of carboplatinum IP. A second mouse was treated with recombinant murine TPO (Zymogenetics Inc, Seattle, WA) at 12,000 U IP daily and a third with TPO vehicle IP daily. All mice were killed after 16 days. Experiment 3 consisted of three mice. One mouse received two daily doses of 100 µL RAMPS (kindly supplied by G. Roth, VAMC, Seattle, WA) IP and was killed after 96 hours. One mouse was treated with 12,000 U recombinant murine TPO IP daily for 7 days and another with TPO vehicle alone for 7 days before death. Experiment 4 consisted of 4 mice treated with daily doses of 175 µL RAMPS IP on day 1 and one untreated control mouse. Two of the mice received 125 µL IP doses on subsequent days. Because the maximal effect of RAMPS on platelets is seen on 6 hours, one mouse was killed daily 6 hours after the RAMPS injection on days 1, 2, and 3. The fourth treated mouse died before the fourth dose of RAMPS could be administered of an acute hemorrhagic diathesis. Of the five mice that remained untreated on these successive dosing days, RNA isolation. Tissues were disrupted in an 11 mol/L guanidium thiocyanate solution using a Polytron (Polytron Drives, Patterson, NJ) tissue homogenizer. Total cellular RNA was obtained using the RNAGents Total Cellular RNA Isolation Kit (Promega Corp. Madison, WI) according to the manufacturer’s directions.

RT-PCR. In batched and parallel reactions, equal amounts of total cellular RNA from each control and treated mouse organ were subjected to reverse transcription with an oligo dT₁₅ primer (Pharmacia Biotech Inc, Piscataway, NJ) using the Superscript II system (GIBCO BRL, Gaithersburg, MD) according to the manufacturer’s specifications. One tenth of the reverse transcription reaction was subjected to PCR in parallel using AmpliTaq (Applied Biosystems, Foster City, CA). A 717-bp fragment was obtained using the oligonucleotides +54 (5'-TCTGTCCAGGCCCGTAGTGC) +73 and +771 (5'-GTTCATCCACAGGTCCGTG) +751. Batched reactions for each reverse transcribed RNA species were separated into 30-µL aliquots, subjected to at least three different numbers of PCR cycles, and size fractionated on agarose gels. These same reverse transcription reactions were subjected to 20 cycles of PCR with limiting concentrations of primers specific for glyceraldehyde-3-dehydrogenase (GAPDH) +570 (5'-CAAGTTGTCTAAGGTACC) +550 and +376 (CCATGGAAGGCTGGG) +394 to serve as an RNA loading and reverse transcription control. The number of PCR cycles were chosen so that the relative intensity of the bands seen on agarose gels would be linearly related to the relative concentration of the TPO-specific mRNA species present in the reverse transcription reactions.

Fig 1. Semiquantitative RT-PCR can detect variations in TPO-specific mRNA levels. RT-PCR performed in parallel as described in the Materials and Methods on liver total cellular RNA diluted 1:5 with RNase-free water (1.5 LIVER) and undiluted liver total cellular RNA (LIVER). Negative and positive control lanes labeled 0 and +, respectively. Arrow marks the major band under study corresponding to TPO 1 and TPO 2; the minor band corresponding to TPO 3 PCR product is seen beneath.

RESULTS

TPO mRNA detected by RT-PCR is predominantly TPO 1. Gurney et al.²² report two TPO isoforms, TPO 2 and TPO 3, that differ from full length TPO 1 by 12-bp and 112-bp deletions, respectively, leading to truncation in the fourth exon. Of eight clones sequenced, six contained sequences consistent with full-length TPO 1 and two were found to contain the 12-bp deletion that characterizes TPO 2. The truncated form, TPO 3, could be differentiated by size using these primer pairs and could only be appreciated after 30 cycles of PCR. The minor band seen in Fig 1 is consistent with TPO 3. Thus, the RT-PCR method on which this study is based predominantly recognizes the active isoform TPO 1 with some minor contribution of the poorly secreted TPO 2 and TPO 3 forms.

Effect of experimental conditions on peripheral blood counts. The results of posttreatment complete blood counts from each set of mice are shown in Table 1. Both radiation and RAMPS treatment regimens resulted in moderate to severe thrombocytopenia, with the most profound decrease seen after successive doses of RAMPS in experiment 4. All concentrations of recombinant TPO (rTPO) used in this study induced substantial thrombocytosis.

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**Table 1. Effect of Experimental Conditions on Peripheral Blood Counts of Study Mice as Measured on Coulter Counter**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Condition</th>
<th>PLT</th>
<th>WBC</th>
<th>HCT</th>
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<td>1335.0</td>
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</tr>
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<td>0.2</td>
<td>5.7</td>
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<tr>
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</tr>
<tr>
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<td>49.0</td>
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<tr>
<td>4 RAMPS 54 h</td>
<td>8.0</td>
<td>0.6</td>
<td>6.0</td>
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Experimental groups as designated in the Methods and Materials section.

Abbreviations: WBC, white blood count per microliter; HCT, hematocrit; PLT, platelet count per microliter.

**Semiquantitative RT-PCR can detect variations in TPO-specific mRNA levels.** The semiquantitative RT-PCR technique used in this study capitalizes on the fact that PCR bands derived from more abundant mRNA species will be seen in lower concentrations. To show the ability of this method to detect differences in mRNA concentrations, we performed the technique on RT-PCR reactions containing transcribed mRNA were present in each PCR reaction. To extend these results, we performed a second experiment, this time including a rTPO-treated animal. Figure 3 confirms earlier results that no significant differences can be discerned in control myelosuppressed or rTPO-treated liver or kidney samples from experiment 2. The presence of more prominent bands in radiated versus control marrow and spleen lanes as well as absent bands in the rTPO-treated marrow and spleen lanes support the conclusion that TPO mRNA can be modulated in response to changes in platelet counts.

**TPO-specific mRNA responds to changes in platelet counts mediated by RAMPS.** To study the regulation of TPO mRNA levels further, we used a second independent method for induction of acute thrombocytopenia. Comparison of the TPO-specific PCR bands from vehicle, rTPO-, and RAMPS-treated mice in Fig 4 again shows that thrombocytopenia failed to alter TPO mRNA levels in liver or kidney. As was seen after myelosuppression-induced thrombocytopenia, a substantial increase in band intensity can be seen in the marrow and spleen of RAMPS-treated mice. In addition, a decrease in band intensity is seen in marrow derived from the rTPO-treated animal. Comparison of control and rTPO-treated spleen is inconclusive given the low intensity of the control band. These results suggest that, in marrow and, to a lesser extent, in spleen, TPO mRNA levels increase in response to acute thrombocytopenia and decrease with thrombocytosis.

The RAMPS-treated mice in experiment 4 achieved the most profound thrombocytopenia of all those tested. Examination of the relative band intensities shown in Fig 5 again

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**Fig 2. TPO-specific mRNA increases after irradiation/carboplatinum therapy.** Results of semiquantitative RT-PCR on liver, kidney, spleen, and marrow-derived total cellular RNA from untreated and carboplatinum/radiation-treated mice as described in the Materials and Methods. Reactions were run for 25, 28, or 31 cycles. The positive control lane is labeled cDNA. GAPDH control reactions after 20 cycles are included for each RNA studied: untreated liver (Le), treated liver (Lx), untreated kidney (Kc), treated kidney (Kx), untreated spleen (Sc), treated spleen (Sx), untreated marrow (Mc), treated marrow (Mx), and negative control (Ø).
Fig 3. TPO-specific mRNA responds to changes in platelet counts mediated by irradiation/carboplatinum (XRT/Carbo) treatment. Results of semiquantitative RT-PCR on liver, kidney, spleen, and marrow-derived total cellular RNA from untreated, RAMPS-, and recombinant mTPO-treated mice as described in the Materials and Methods. Reactions were run for 25, 28, or 31 cycles. Negative control (0) and positive control lanes (cDNA) are shown. GAPDH control reactions after 20 cycles included for each RNA sample: untreated liver (L), XRT/Carbo-treated liver (Lt), untreated kidney (Kc), XRT/Carbo-treated kidney (Kx), TPO-treated kidney (Kt), untreated spleen (Sc), XRT/Carbo-treated spleen (Sx), TPO-treated spleen (St), untreated marrow (Mc), XRT/CTx-treated marrow (Mx), TPO-treated marrow (Mt), and negative control (0).

shows that no changes in TPO-specific mRNA can be detected in liver or kidney derived lanes, but that bands show increased intensity in RAMPS-treated marrow and spleen. Although this effect can be seen as early as 6 hours after RAMPS administration, it is most apparent after 30 and 54 hours of acute thrombocytopenia.

RAMPS treatment does not affect marrow cellularity or composition. In the present study we used two well-established methods to induce severe thrombocytopenia, i.e., myelo-suppressive therapy and antiplatelet antisera. These methods may affect the cellular composition of spleen and marrow; thus, it is possible that the induction of thrombocytopenia may have altered the relative proportion of TPO-producing cells in these organs. The end result could be an artificial enrichment for TPO mRNA. RT-PCR surveys of primary cell cultures and of cell lines performed in our laboratory have shown that stromal elements are a source of TPO-specific mRNA in marrow, suggesting that relative enrichment could possibly introduce a sampling bias. Because the same results were seen with either radiation of RAMPS induction of thrombocytopenia, it became important to know whether these manipulations altered the cellularity or composition of marrow. Figure 6 shows photomicrographs of humeri taken from the same mice used in experiments 1 and 4. Compared with the normally 100% cellular marrow of the control mouse in Fig 6A, the myelosuppressed mouse marrow was hypocellular, with a predominance of marrow stromal cells, histiocytes, and plasma cells (Fig 6B). As expected, the administration of rTPO resulted in substantial megakaryocyte expansion (Fig 6C). However, examination of the humeri taken from each of the RAMPS-treated mice shown in Fig 6D through F showed no change in overall cellularity or cellular composition compared with the control mouse. On the marrow corresponding to the 54 hours of RAMPS exposure, which is when the biologic effect of TPO is first seen in various biologic and marrow colony assays,
Liver  Kidney  Spleen  Marrow

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>6 Hrs RAMPS</th>
<th>30 Hrs RAMPS</th>
<th>54 Hrs RAMPS</th>
</tr>
</thead>
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<tr>
<td>Lc L6 L30 L54</td>
<td>Kc K30 K56</td>
<td>Sc S6 S30 S54</td>
<td>Mc M6 M30 M54</td>
</tr>
</tbody>
</table>

Fig 5. TPO mRNA increases can be detected 6 hours after RAMPS administration. Results of semiquantitative RT-PCR on liver, kidney, spleen, and marrow-derived total cellular RNA from untreated and RAMPS-treated mice as described in the Materials and Methods. Reactions were run for 20, 25, or 30 cycles. Negative control (Ø) and positive control lanes (cDNA) are shown. GAPDH control reactions after 20 cycles are included for each RNA sample: untreated liver (Lc), 6-hour RAMPS-treated liver (L6), 30-hour RAMPS-treated liver (L30), 54-hour RAMPS-treated liver (L54), untreated kidney (Kc), 6-hour RAMPS-treated kidney (K6), 30-hour RAMPS-treated kidney (K30), 54-hour RAMPS-treated kidney (K54), untreated spleen (Sc), 6-hour RAMPS-treated spleen (S6), 30-hour RAMPS-treated spleen (S30), 54-hour RAMPS-treated spleen (S54), untreated marrow (Mc), 6-hour RAMPS-treated marrow (M6), 30-hour RAMPS-treated marrow (M30), and 54-hour RAMPS-treated marrow (M54).

a substantial increase in megakaryocyte number and size can be seen (Fig 6F). These data would indicate that the increase in TPO-specific mRNA after RAMPS-induced thrombocytopenia is not due to a gross change in the composition or cellularity of the marrow.

TPO effects on marrow composition and cellularity. From the photomicrograph of marrow derived from the rTPO-treated mouse seen in Fig 6, substantial expansion of the megakaryocyte compartment without change in overall cellularity is evident. On these animals, the megakaryocyte mass accounts for nearly 30% of the marrow space. Because these cells contribute a significant proportion of the mRNA in these tissues, it also became important to know whether megakaryocytes contained TPO-specific mRNA. Using the same experimental conditions used in this study, TPO mRNA could not be detected from purified megakaryocytes despite 80 cycles of RT-PCR (data not shown). However, this implies that the reduction in band intensity seen in the marrow and spleen from rTPO-treated animals might be explained, at least in part, by relative dilution from megakaryocyte-derived mRNA.

DISCUSSION

The gene for the Mpl ligand, TPO, has recently been cloned by a number of groups. Work by Wendling et al. has shown that virtually all of the thrombopoietic activity of thrombocytopenic plasma can be accounted for by the Mpl ligand, thereby supporting earlier studies that suggest that TPO protein levels are inversely related to platelet counts. In this study, we sought to determine whether TPO-specific mRNA levels vary in tissues and could therefore account for changes in serum TPO concentration. To do so, we developed a semiquantitative RT-PCR-based assay that could easily detect TPO-specific transcripts in liver, kidney, spleen, and marrow. Because mRNA was made from whole tissue extracts, it is impossible for us to determine from this study which cells in these tissues are the site of TPO production. It is possible that a common cell type found in all these tissues, such as endothelial cells, fibroblasts, or tissue macrophages, is the source of TPO production. Surveys of several cell lines, including primary and transformed endothelial cells and fibroblasts, have shown them to express TPO message and protein. Although some inferences can be made from RT-PCR in cell lines, the cellular localization of TPO awaits the completion of in situ hybridization experiments, which are already underway.

Two models of TPO gene regulation have been proposed. In the first, serum levels are maintained solely by platelet uptake and metabolism of TPO. In the second, platelet levels are sensed, resulting in appropriately increased or decreased levels of TPO gene expression. The phenotype of a recently reported NF-E2 knockout mouse lends credence to the assertion that platelet metabolism of TPO is not the sole determinant of serum TPO levels. In the NF-E2–/– phenotype, although platelet counts are low, serum TPO levels remain normal. TPO levels in these transgenic mice are therefore discordant with those expected from a purely catabolic model. The precise impact of the NF-E2–/– phenotype on the modulation of TPO mRNA levels in response to thrombocytopenia cannot be determined completely, whereas no difference in liver TPO mRNA levels was seen between control and mutant mice; spleen- and marrow-derived RNA was not tested in their study.
Further support for an inductive model of TPO gene regulation can be found in the observations of Wendling et al.\textsuperscript{23} in RAMPS-treated mice. Whereas a modest increase in serum TPO activity could be detected at the time of platelet nadir in a BAF3/mpl proliferation assay, peak serum activity was seen at 24 hours after RAMPS administration. These same observations were also reported by Hunt et al.\textsuperscript{24} In a murine model of immune-mediated thrombocytopenia, peak serum TPO levels were detected not at the time of platelet nadir, which occurred at 8 hours, but rather at 24 hours after treatment. From their findings, Hunt et al.\textsuperscript{24} considered the delayed activity peak to represent the time required for TPO gene induction, as well as TPO protein production and accumulation. This response profile is consistent with the induction of TPO-specific mRNA in the RAMPS-treated mice reported in the present study.

The results reported here indicate at least one molecular basis for TPO regulation. The semiquantitative method used in this study shows that differences in mRNA levels detected in some organs must be due to either transcriptional or post-transcriptional regulatory mechanisms. From Northern blot analysis, it is obvious that the liver and the kidney are the
two most predominant sites of TPO mRNA. However, these two tissues do not exhibit modulation of TPO mRNA in response to changes in platelet counts in our study. In the spleen and marrow, perhaps the more physiologically relevant tissues for thrombopoiesis, TPO-specific mRNA levels were found to be inversely related to platelet counts. Although this does not exclude a role for platelet catabolism in maintaining appropriate serum TPO levels, it does provide evidence for an alternative mechanism regulating TPO in serum in response to changes in platelet counts. Thus, at least in marrow and spleen, mRNA is either transcriptionally induced or stabilized. However, the magnitude by which TPO mRNA levels change in both spleen and marrow cannot be assessed for comparison with the degree by which TPO protein activity and levels change in serum. Correlation of serum TPO activity and serum and cellular protein levels with mRNA production from these tissues is required to address quantitative aspects of TPO expression. It is anticipated that the modulation of mRNA levels seen in mouse spleen may not be seen in human splenic tissues because of the greater functional role of the spleen as a site of hematopoiesis in the mouse. Nonetheless, these findings in mice can likely be extrapolated to human marrow.

Studies of the mRNA isoforms reported by Gurney et al. have shown that only the full-length TPO mRNA is secreted and accounts for the serum TPO activity seen in thrombocytopenia. It is important to note that the majority of mRNA can likely be extrapolated to human marrow. From the results obtained in this study some generalities can be made about the time course of the TPO mRNA response to thrombocytopenia. From experiment 4, in which mice were killed 6 hours after each successive dose of RAMPs, it appears that a small but discernible increase in TPO mRNA can be detected in spleen and marrow as early as 6 hours after the onset of acute thrombocytopenia. This increase continues to 30 hours and, although no difference can be seen in this study between 30 and 54 hours after the induction dose of RAMPs, the band intensity remains higher than control throughout this time period. Data from the myelosuppressed mice that were killed after day 16 showed an increased level of TPO mRNA, suggesting that, in this model, TPO mRNA remains high in marrow and spleen as long as the platelet count remains low. Thus, although the response of TPO mRNA in marrow and spleen is fairly rapid, by 6 hours, with peak levels by 30 hours, it is also long-lasting, suggesting an ongoing signal that results in higher TPO mRNA levels. Likewise, the reduction in TPO mRNA levels in response to exogenously administered rTPO-induced thrombocytosis can be seen for 16 days. Although it is not certain that this is due solely to modulation of mRNA levels or due to relative dilution of TPO mRNA by increased amounts of megakaryocyte RNA, it is consistent with the data from the thrombocytopenic animals. This issue can be further addressed by studying mice hypertransfused with platelets to avoid disturbing the megakaryocyte compartment of the marrow. Further studies examining the effect of platelet recovery after thrombocytopenia on TPO mRNA levels in this myelosuppressed model would also be informative. The nature or the source of the signal that allows tailoring of TPO mRNA levels to platelet demand is not clear from our data and is the basis of ongoing study.

It is also apparent from examination of the peripheral blood counts performed on the mice from these studies that, in addition to thrombocytopenia, carboplatinum/radiation as well as RAMPs treatment affects the peripheral red blood cell and white blood cell compartments. Because several additional cytokine levels would be expected to be elevated in these animals (eg, erythropoietin, GM-CSF, and G-CSF), it is possible that the stimulus for TPO mRNA induction may not be due to isolated thrombocytopenia alone. However, in the RAMPs-treated mouse of experiment 1, no effect is seen on either the white blood cell count or hematocrit. Thus, it appears that TPO mRNA can be induced in the absence of anemia or leukopenia.

This study provides an initial insight into the molecular mechanisms that regulate expression of the TPO gene. Current and future efforts are directed at more precise quantitation and the cellular localization of TPO mRNA. The development of in vitro models of TPO production and studies including nuclear run-on assays and mRNA half-life determinations will be required to confirm the constitutive nature of TPO expression in liver and kidney and to determine relative contributions of transcriptional and posttranscriptional mechanisms that regulate TPO mRNA levels in response to changes in platelet mass. It is hoped that a better understanding of the molecular processes that regulate this important mediator of thrombopoiesis will lead to greater insights into the process of hematopoiesis as a whole.

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