Thrombopoietin in Thrombocytopenic Mice: Evidence Against Regulation at the mRNA Level and for a Direct Regulatory Role of Platelets

By Ruedi Stoffel, Adrian Wiestner, and Radek C. Skoda

Thrombopoietin (TPO), originally described as an activity in the serum of thrombocytopenic animals that leads to increased production of platelets, has recently been isolated and cloned. Its closest relative in the cytokine superfamily, erythropoietin (EPO), is transcriptionally regulated during anemia, and it was expected that TPO would similarly be regulated during thrombocytopenia. We induced thrombocytopenia in mice and confirmed that TPO activity was upregulated, as determined by a bioassay. Liver and kidney were found to be the major sources of TPO mRNA. Surprisingly, TPO mRNA in these tissues was not upregulated in thrombocytopenic mice. Using a sensitive RNase protection assay that can distinguish between TPO isoforms, we found no change in the profile of mRNA for these isoforms. A semi-quantitative reverse transcription-polymerase chain reaction assay also did not demonstrate upregulation of TPO mRNA in the spleen. Thus, the increase of TPO activity during thrombocytopenia is not caused by regulation at the level of TPO mRNA. Furthermore, isolated mouse platelets absorbed high amounts of bioactive TPO out of TPO-conditioned medium in a dose-dependent fashion. Our results are consistent with TPO protein being regulated at a posttranscriptional level and/or directly through absorption and metabolism by platelets.

From the Department of Pharmacology, Biozentrum of the University of Basel, Basel; and the Division of Hematology, Department of Research, University Hospital, Basel, Switzerland.

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Address reprint requests to Radek C. Skoda, MD, Department of Pharmacology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

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performed in 50-mmol KCl, pH 8.3, 0.25 mmol/L deoxynucleotide triphosphates (dNTPs), 0.75 mmol/L MgCl₂, 20 mmol/L Tris pH 8.3, 0.25 mmol/L deoxynucleotide triphosphates (dNTPs), 0.75 mmol/L of each primer, and 0.05 U/μL Taq polymerase (Life Technologies, Gaithersburg, MD). The primer pair for mouse TPO was 5'-GTCTATCCCTG'ITCTG-3' (forward) and 5'-CAACAA-ncologies, Gaithersburg, MD). The primer pair for mouse TPO was 5'-CACAGGACTAGAACACCTGC-3' (reverse), amplifying a 249-bp fragment that can be detected for TPO-2. If genomic DNA was present in the RNAs, fragments of 168, 160, and 72 nt would be generated, because two introns are present in the genomic region spanned by this riboprobe. RNA loading was normalized with a riboprobe for mouse hypoxanthine-guanine phosphoribosyl transferase (HPRT), a housekeeping gene. A Sca I-HindIII fragment representing nucleotides 679 to 840 of mouse HPRT cDNA was subcloned into pBluescript. This riboprobe protects a 161-nt fragment. HPRT riboprobe was mixed with the TPO probe and added as an internal standard to each sample. Protected fragments were separated on 6% polyacrylamide/8 mol/L urea sequencing gels. Dried gels were exposed on film or on phosphorimager screens, and quantities of radioactive bands were performed on a PhosphoImager 425 using the ImageQuant software (Molecular Dynamics Inc, Sunnyvale, CA).

For RT-PCR analysis, oligo(dT)-primed first strand cDNA was synthesized from 2 μg total RNA using RNaseH⁻ MuLV reverse transcriptase (Stratagene), in a reaction volume of 20 μL under conditions recommended by the manufacturer. This reaction mixture was diluted to 50 μL with H₂O, heated to 95°C for 5 minutes to inactivate reverse transcriptase, and then quickly chilled on ice for 10 minutes. PCR was performed with 2 μL of the first strand cDNA as a template in a final reaction volume of 20 μL. The reaction was performed in 50-mmol/L KCl, 1.5 mmol/L MgCl₂, 20 mmol/L Tris pH 8.3, 0.25 mmol/L deoxynucleotide triphosphates (dNTPs), 0.75 μmol/L of each primer, and 0.05 U/μL Taq polymerase (Life Technologies, Gaithersburg, MD). The primer pair for mouse TPO was 5'-GTCTATCCCTG'ITCTG-3' (forward) and 5'-CAACAA-ncologies, Gaithersburg, MD). The primer pair for mouse TPO was 5'-CACAGGACTAGAACACCTGC-3' (reverse), amplifying a 249-bp product.²⁷ We performed 30 cycles for TPO and 28 cycles for HPRT, each consisting of 60 seconds at 94°C, 60 seconds at 55°C, and 60 seconds at 72°C using a DNA thermal cycler (Perkin Elmer, Norwalk, CT). The PCR products were electrophoresed on 1.5% agarose gels and then transferred to Hybond N+ membrane (Amersham, Buckinghamshire, UK). Blots were probed with internal 3ZP-labeled HPRT riboprobe and added as an internal standard to each sample. Protected fragments were separated on 6% polyacrylamide/8 mol/L urea sequencing gels. Dried gels were exposed on film or on phosphorimager screens, and quantities of radioactive bands were performed on a PhosphoImager 425 using the ImageQuant software (Molecular Dynamics Inc, Sunnyvale, CA).

RESULTS

To examine the effects of thrombocytopenia on the levels of TPO mRNA, we injected mice intraperitoneally with 0.1 mL of rabbit anti-mouse platelet serum (RAMPS). Groups of three mice were killed at various times and analyzed (Fig 1A). After 4 hours, the platelet counts decreased more than 40-fold to a mean of 22 × 10⁴/μL and remained low at 24 and 48 hours. Two of the three mice killed after 24 hours had normal platelet numbers (not shown). These two nonresponders were not further examined. To assure that the animals were killed at the relevant time, we also determined TPO activity in conditioned media. We used a proliferation assay with BaF3/ mpl cells that express the mouse c-mpl and proliferate in response to TPO. Only the transfected BaF3/ mpl cell line responded to the sera from thrombocytopenic mice, but not the parental untransfected BaF3 cells. Proliferation of BaF3/ mpl cells was measured by incorporation of ³H-thymidine (Fig 1A). The values for the untreated controls were indistinguishable from background incorporation into BaF3/ mpl cells in TPO-free medium. Thus, serum TPO concentrations in normal mice are below the limit of detection of this assay. TPO increased to measurable levels after 24 hours and was elevated at 24 and 48 hours. RAMPS does not lead to destruction of megakaryocytes.³¹ This was confirmed by cyto- spin analysis of bone marrow cells and histopathology of the spleens from these mice (not shown). The average numbers of megakaryocytes per spleen section increased from 30 in the controls, and 33 at 4 hours, to 52 at 24 hours, and to 109 at 48 hours (not shown).

To examine if megakaryocyte mass is important for the regulation of TPO mRNA, we also analyzed mice pancytopenic after TBI with 8 Gy. Platelet levels in mice treated with

16 hours in medium without IL-3, and plated in 96-well plates at 10⁴ cells per well in 100 μL of medium containing dilutions of mouse serum. After 22 hours, 1 μCi of ³H-thymidine was added to each well, and incorporation of ³H-thymidine was measured after 6 hours in a β-counter. Alternatively, XTT, a colorimetric tetrazolium dye,²⁹ was used to determine TPO activity in conditioned media. Cells (5 × 10⁴ per well) were seeded, and after 3 days of stimulation, 50 μL of a 1 mg/mL stock solution of XTT with 5 mmol/L phenazine methosulfate (PMS), an electron coupling agent, was added to each well. The product of XTT reduction by viable cells, reflecting the number of cells per well, was measured at 4 hours at 450 nm. A stable cell line producing mouse TPO was generated by electroporating NIH/3T3 cells with a pG-D-TPO expression construct followed by selection in 0.5 mg/mL G418 as above. TPO-secreting clones were identified using the TM17 proliferation assay.

Adsortion of TPO by platelets. Mouse platelets were isolated from five mice as described.³⁶ The platelets were washed with PBS and counted in a Neubauer chamber. The automated platelet count of the purified platelet preparation was 166 × 10⁹/μL, with no detectable white blood cells (WBCs) and no red blood cells (RBCs). In the stained cytopsin of this purified platelet preparation with approximately 500,000 platelets, we counted a total of 108 RBCs and no WBCs. Specified numbers of platelets were then pelleted for 5 minutes at 1,300g and incubated while rotating for 1 hour at 37°C with 50 μL of conditioned medium containing TPO or IL-3. Platelets were removed by 5 minutes of centrifugation at 1,300g, and TPO activity of the supernatants was assessed by the BaF3/ mpl cell proliferation assay.
TBI decreased slowly and reached a mean of $43 \times 10^8/\mu L$ at day 9 (Fig 1B). Conversely, TPO levels were measurable at day 6 and increased further at day 9. No megakaryocytes were found on histopathologic examination of spleens at days 6 and 9 (not shown).

We devised a sensitive RNase protection assay that can distinguish between four TPO isoforms (Fig 2). The TPO riboprobe spans the region between nucleotides 330 and 728 of the mouse TPO cDNA sequence.\textsuperscript{11} RT-PCR from mouse liver RNA yielded the full-length TPO cDNA (TPO-1) and two shorter isoforms, TPO-2 and TPO-3, that have been described previously.\textsuperscript{32,33} In addition, we found a fourth isoform, TPO-4, which has a deletion of 197 bp from position 569 to 766 of the mouse TPO cDNA.\textsuperscript{11} We analyzed expression of the TPO isoforms and found the highest levels in liver and kidney, which express the highest levels of TPO (Fig 2), and smaller amounts in brain and testes.

We have analyzed TPO mRNA levels during thrombocytopenia in mice and have found no upregulation despite a measurable increase of TPO activity in serum (Fig 1). EPO, the closest homologue of TPO, can be upregulated more than 100-fold at the mRNA level,\textsuperscript{18,20} and it was suspected that a similar mechanism would exist for TPO. Liver and kidney, which express the highest levels of TPO (Fig 2), were examined by an RNase protection assay (Fig 3). With the same RNase protection assay, we tested the possibility that TPO isoforms, which are believed to be generated by alternative or aberrant splicing, might play a role in the

**DISCUSSION**

We have analyzed TPO mRNA levels during thrombocytopenia in mice and have found no upregulation despite a measurable increase of TPO activity in serum (Fig 1). EPO, the closest homologue of TPO, can be upregulated more than 100-fold at the mRNA level,\textsuperscript{18,20} and it was suspected that a similar mechanism would exist for TPO. Liver and kidney, which express the highest levels of TPO (Fig 2), were examined by an RNase protection assay (Fig 3). With the same RNase protection assay, we tested the possibility that TPO isoforms, which are believed to be generated by alternative or aberrant splicing, might play a role in the
regulation of TPO activity (Fig 3A and B). Two TPO isoforms have been described. TPO-2 has a deletion of the four amino acids LPPQ at position 112 to 115, and TPO-3 is produced by an internal splice in the last exon. The proteins for TPO-2 and TPO-3 were expressed but not secreted by transfected cells lines, suggesting that these proteins are retained in the secretory pathway. As these isoforms are conserved between humans, pig, and mouse, it was suspected that they might play a regulatory role. Interestingly, no splice variants have been described for EPO, which shares a highly homologous gene structure with TPO. However, we found no changes in mRNA levels for TPO-2 or TPO-3 during thrombocytopenia (Fig 3A and B). Therefore, regulation of TPO does not occur at the mRNA level in liver and kidney and does not involve changes in the ratios of TPO isoform mRNAs.

Because we found no expression of TPO in spleen and bone marrow by RNase protection (Fig 2) and these organs are the sites of megakaryopoiesis in mice, upregulation of TPO produced in situ in a paracrine fashion could have a major effect on megakaryopoiesis. By RT-PCR, we were able to detect TPO mRNA in spleen (Fig 4) and, less reliably, also in bone marrow (not shown). However, there was no significant increase of TPO mRNA detectable by RT-PCR (Fig 4). Although this assay is not as quantitative as RNase protection, we should have been able to detect a 5- to 10-fold mRNA increase. Plasma from thrombocytopenic animals was sufficient to induce accelerated platelet production, and purified thrombopoietin caused an up to fivefold increase in platelet count when injected into normal recipients. This demonstrates that TPO is a potent humoral stimulator. As the levels of expression were very low and we could not detect upregulation, the physiologic importance of locally produced TPO in the spleen remains unclear.

It has been proposed that platelets might be directly involved in the regulation of circulating TPO activity. Platelets express mpl protein. Purified fractions of megapoietin, which was shown to be identical with TPO, lost activity when first incubated with sheep platelets and tested after removal of platelets by centrifugation. Megapoietin activity was assessed measuring increase in megakaryocyte ploidy of isolated rat megakaryocytes. We used the proliferation assay with BaF3/mpl cells to more directly measure TPO exposed to various concentrations of isolated mouse platelets (Fig 5). When TPO-conditioned media were exposed to 1 × 10⁹ platelets per microliter, the optical density 450-nm reading, reflecting the number of proliferating BaF3/mpl cells, decreased by 40% (Fig 5). As in this range the dose response curve of the assay is linear with the logarithm of the TPO

### Table 1. Relative Amounts of TPO Isoforms in Mouse Tissues

<table>
<thead>
<tr>
<th>TPO-1</th>
<th>Liver</th>
<th>Kidney</th>
<th>Testis</th>
<th>Brain</th>
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<tr>
<td>100</td>
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<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>TPO-2</td>
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<td>52</td>
<td>28</td>
<td>28</td>
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<td>TPO-3</td>
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<td>TPO-4</td>
<td>9</td>
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Radioactive bands from Fig 2A and B were quantified with a phosphorimager. The relative abundance of TPO isoforms is expressed as percent of TPO-1 for each organ. The values were normalized with the number of uridines in the protected fragments. Values for TPO-4 were calculated by subtracting TPO-2 from TPO-2 + 4.
concentration, this represents removal of more than 40% of TPO activity. Using the more sensitive \(^3H\)-thymidine assay, we measured an incorporation in the range of 6,000 cpm with thrombocytopenic sera (Fig 1) and 60,000 cpm with saturating concentrations of 5% TPO-conditioned medium (not shown). Because the values measured with our thrombocytopenic sera are in the region where the curve becomes nonlinear, we cannot accurately assess the TPO concentration. However, we can make an approximate estimate and find that our TPO-conditioned medium at 2.5% contained at least a 100-fold excess of TPO activity compared with thrombocytopenic sera. These results demonstrate the ability of platelets to downregulate the free serum TPO concentration. The capacity of mouse platelets to adsorb TPO in our assay appears to be higher than the calculated binding capacity of sheep platelets.\(^{21}\) However, these results are not directly comparable, because the assays used to measure TPO were not the same. Isolated platelets may be activated and display a higher binding than platelets under physiologic conditions.

We show that the increase in TPO activity during thrombocytopenia is not mediated by changes in TPO mRNA abundance. Our data is consistent with TPO regulation at a translational or posttranslational level and/or with regulation of platelet production.
directly through metabolism by platelets. A number of other steps of TPO biosynthesis and metabolism may be important as well, as will be determined through detailed studies of the TPO protein in the future.

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REFERENCES

20. Goldberg MA, Gaut CC, Bunn HF: Erythropoietin mRNA levels are governed by both the rate of gene transcription and post-transcriptional events. Blood 77:271, 1991
tion by acid guanidium thiocyanate-phenol-chloroform extraction.
Anal Biochem 162:156, 1987
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