Regulation of the Serum Concentration of Thrombopoietin in Thrombocytopenic NF-E2 Knockout Mice

By Ramesh A. Shivdasani, Paul Fielder, Gilbert-Andre Keller, Stuart H. Orkin, and Frederic J. de Sauvage

The mechanisms that regulate circulating levels of thrombopoietin (Tpo) are incompletely understood. According to one favored model, the rate of Tpo synthesis is constant, whereas the serum concentration of free Tpo is modulated through binding to c-Mpl receptor expressed on blood platelets. Additionally, a role for c-Mpl expressed on megakaryocytes is suggested, particularly by the observation that serum Tpo levels are not elevated in human immune thrombocytopenic purpura. Whereas direct binding of Tpo to platelets has been demonstrated in vitro and in vivo, the role of megakaryocytes in modulating serum Tpo levels has not been addressed experimentally. The profoundly thrombocytopenic mice lacking transcription factor p45 NF-E2 do not predict the increased serum Tpo concentration. Therefore, the radioligand is increased in hematopoietic tissues, where it is detected in association with megakaryocytes and small, abnormal, platelet-like particles found within or close to macrophages. These findings implicate the combination of megakaryocytes and the latter particles as a sink for circulating Tpo in NF-E2 knockout mice, and provide an explanation for the lack of elevated serum Tpo levels in this unique animal model of thrombocytopenia.

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Materials and Methods

Knockout mice and histologic analysis. Mice lacking p45 NF-E2 or c-Mpl were generated and maintained as previously described. 6,14 Control (wild-type) mice were littermates in each case. Spleen and bone marrow were harvested from adult mice, preserved in neutral buffered 10% Formalin, processed for routine histologic analysis with 10-μm sections, and stained with hematoxylin-eosin.

Measurement of serum Tpo levels. This was performed as described previously, using Tpo-dependent modified BaF/3 cells.10

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Detection of c-Mpl protein in platelet-rich plasma. Whole mouse blood collected in EDTA was first centrifuged at 300g to pellet red blood cells and leukocytes. The supernatant was then centrifuged at 1,000g, and the resulting platelet pellet was washed twice in EHS (150 mM NaCl, 1 mM EDTA, and 10 mM HEPES, pH 7.6). The pellet was then lysed in sodium dodecyl sulfate (SDS) sample buffer, and its protein components were resolved by SDS–polyacrylamide gel electrophoresis followed by blotting onto nitrocellulose. Western analysis was performed by standard techniques using a hamster anti-mouse c-Mpl monoclonal antibody and horseradish peroxidase–conjugated goat anti-hamster IgG (Jackson ImmunoResearch Labs, West Grove, PA). The reaction was detected using an ECL kit (Amersham Inc, Arlington Heights, IL) according to the manufacturer’s recommended protocol, followed by autoradiographic exposure for 15 seconds.

In vivo distribution of 125I-Tpo. Recombinant murine (rm)Tpo was iodinated by the indirect iodogen method, purified by size-exclusion chromatography, and formulated in 10 mM Tris, 0.15 mM NaCl, and 0.01% Tween-20, pH 7.4. Because of the poor survival rate of NF-E2-null mice, we were unable to obtain enough adult mice at one time; therefore, two separate groups of mice were used for independent experiments performed at different times using different 125I-rmTpo preparations (each experiment with two wild-type and two NF-E2-null adult mice). Mice were injected intravenously with 0.1 mL of the radioligand formulation (~3.21 μCi) via a lateral tail vein and killed 3 hours later, when the following tissues were collected: experiment 1, blood (in 0.38% citrate), spleen, and sternum; and experiment 2, blood (in 0.38% citrate), spleen, sternum, lung, femur, liver, and kidney. Tissues from all animals were sectioned, rinsed in phosphate-buffered saline, blotted dry, weighed, and stored on ice until aliquots were counted for 1 minute in a Wallac 1470 gamma counter. Values were normalized for tissue weight (cpm per gram) and expressed as the percent of injected dose per gram tissue.10 Sternums and spleens were cut into 2-mm pieces, fixed in Karnovsky’s fixative, and processed for electron microscopic autoradiography.18

RESULTS

Serum Tpo levels. Tpo-dependent cultured cell lines provide a reproducible bioassay for detection and relative quantitation of Tpo in murine serum.16,19 In our assay using c-Mpl±transfected BaF/3 cells,16 the Tpo concentration in normal mouse serum is not detectable, whereas elevated levels associated with thrombocytopenia provide a readily measurable signal. Although the minimum amount of murine Tpo detectable in this assay (ie, its sensitivity) is unknown, the effective range for human Tpo in the assay is 7 to 114 pg/mL. As previously reported,6 Tpo levels in thrombocytopenic c-Mpl±deficient mice are consistently elevated over control levels (Fig 1), and the assay can detect a 300-fold dilution of serum from these mice. In contrast, adult mice homozygous for a disrupted p45 NF-E2 allele14 do not have high circulating Tpo levels, despite apparently more severe thrombocytopenia. Because Tpo levels in surviving adult NF-E2-null mice may reflect a steady state established through unknown physiologic mechanisms, we also examined sera from newborn mice with clinical signs of hemorrhage and noted no increase in the Tpo concentration (Fig 1). These data provide independent confirmation of our previous report that free Tpo is undetectable in the serum of NF-E2 knockout mice by this bioassay.14

Fate of radiolabeled Tpo in vivo. Intravenous injection of radioiodinated Tpo in normal mice results in rapid binding to circulating platelets, followed by clearance of the radiolabel and degradation of the polypeptide; these events occur either more slowly or not at all in mice lacking c-Mpl.10 Thus, c-Mpl protein expressed on the surface of circulating platelets appears to be an important regulator of serum Tpo levels. Figure 2 and Table 1 show the results of simultaneous quantitation of Tpo in murine serum.16,19 In our assay using Tpo-dependent BaF/3 cells. Results are expressed as incorporated 3H-thymidine counts per well after 2 days of culture in medium supplemented with the mouse serum. rTPO, recombinant Tpo (saturating concentration, 10 ng/mL) used as an internal standard.

![Graph](https://via.placeholder.com/150)

**Fig 1.** Relative serum concentrations of Tpo from wild-type, NF-E2-null, and c-Mpl knockout (–/–) mice at various ages as determined in the bioassay using Tpo-dependent BaF/3 cells. Results are expressed as incorporated 3H-thymidine counts per well after 2 days of culture in medium supplemented with the mouse serum. rTPO, recombinant Tpo (saturating concentration, 10 ng/mL) used as an internal standard.
control mice (Fig 2D), which suggests that they remove much more \(^{125}\text{I}\)-Tpo from the circulation than the latter.

One potential reason for the normal level of free Tpo in NF-E2 knockout mice is that the ligand is bound to soluble receptors or circulating megakaryocyte fragments that do not precipitate with the blood cell fraction in the experiment. However, plasma-associated radioactivity in the mutant animals is not increased (Fig 2B). When we subject the pellet resulting from high-speed centrifugation of platelet-rich plasma to immunoblot analysis to assess c-Mpl protein levels, control samples generate a strong signal, whereas equal amounts of the identical fraction from NF-E2\(^{+/−}\) plasma yield a substantially weaker one (Fig 3). The absence of recognizable platelet forms in the peripheral blood of mutant animals suggests that the source of this small amount of intact c-Mpl protein within an insoluble plasma fraction may be circulating megakaryocyte fragments. Moreover, immunoreactive c-Mpl is undetectable in the soluble plasma fraction of control or NF-E2\(^{+/−}\) adult mice (data not shown). Taken together, these results make it unlikely that free Tpo in NF-E2 knockout mice is cleared largely through mechanisms operating within the circulation.

**Binding of \(^{125}\text{I}\)-Tpo to NF-E2\(^{−/−}\) megakaryocytes and megakaryocyte fragments in vivo.** Although blood platelets, which carry approximately 200 c-Mpl molecules per cell in mice,\(^ {10}\) are likely to be the major source of this receptor, it is also expressed on megakaryocytes. Loss of NF-E2 in vivo is accompanied by megakaryocytosis,\(^ {14}\) marrow hypercellularity, and splenomegaly.\(^ {20}\) Indeed, prior studies may have underestimated the degree of megakaryocyte hyperplasia in the steady state; extensive review of spleen and bone marrow histologic sections reveals considerable expansion of the megakaryocyte population in adult mice (Fig 4). Thus, a substantial amount of c-Mpl may be available on NF-E2\(^{−/−}\) megakaryocytes to sequester free Tpo from the circulation.

Figure 2C and Table 1 show that a greater fraction of

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**Table 1. Comparison of Tissue-Associated Radioactivity in Wild-Type and NF-E2-Null Mice**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Wild-Type (%)</th>
<th>NF-E2-Null (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>40.0 ± 0.64</td>
<td>19.9 ± 2.97</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.31 ± 0.33</td>
<td>6.64 ± 0.21</td>
</tr>
<tr>
<td>Femur</td>
<td>0.38 ± 0.09</td>
<td>0.58 ± 0.07</td>
</tr>
<tr>
<td>Sternalur</td>
<td>0.33 ± 0.22</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>Lung</td>
<td>4.24 ± 0.85</td>
<td>0.94 ± 0.04</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.31 ± 0.19</td>
<td>1.23 ± 0.31</td>
</tr>
<tr>
<td>Liver</td>
<td>3.69 ± 0.47</td>
<td>3.66 ± 0.53</td>
</tr>
</tbody>
</table>

Data (mean ± SD) are presented as a % of the total dose per organ; n = 2.
to platelets (Fig 6B). Surprisingly, we also observe binding of the radioligand to previously undetected platelet-like particles in the spleen of NF-E2<sup>0/0</sup> adult mice; most of these are small, found within or in close contact with macrophages, and contain few or no granules (Fig 6A). However, as in the bone marrow, numerous silver grains are also seen in association with megakaryocytes within NF-E2-null and control spleens (data not shown). Taken together, these data suggest that NF-E2<sup>0/0</sup> megakaryocytes release abnormal fragments or platelet-like particles that do not circulate in appreciable numbers but are cleared rapidly by splenic macrophages. The presence of these particles is consistent with the previously noted abnormal megakaryocyte maturation and the sublethal bleeding in rare NF-E2<sup>0/0</sup> mice that survive beyond the neonatal period. Binding of Tpo to these particles and to megakaryocytes provides an explanation for the absence of increased levels of free Tpo in this animal model of thrombocytopenia.

**DISCUSSION**

Whereas the relative roles of various cytokines in megakaryocytogenesis continue to be investigated, Tpo is clearly the major physiologic regulator of platelet production in vivo.<sup>21,22</sup> Therefore, it is important to understand how the serum concentration of Tpo is controlled. Experimental manipulation of the platelet mass in animals has provided invaluable insights; immune or chemically induced thrombocytopenia results in a detectable increase in serum Tpo activity, which can be abrogated by transfusion of platelets or absorbed by incubation with platelets in vitro.<sup>9,23</sup> This suggests that platelets are the primary regulators of the concentration of free Tpo. A likely similar role for megakaryocytes has been inferred,<sup>11,13,15</sup> but has not received direct experimental support. Megakaryocytes have access to the circulation by virtue of a parasinusoidal location in hematopoietic tissues and their intravascular cytoplasmic processes.<sup>21,23</sup> In addition, each megakaryocyte presents a
large surface area, which is comprised not only of the plasma membrane but also of that fraction of the demarcation membrane system known to be in communication with the cell exterior. These considerations add plausibility to a role for megakaryocyte-associated c-Mpl in sequestering Tpo from the circulation.

To explore the reasons for apparently normal (ie, undetectable in murine bioassays) serum levels of Tpo in the profoundly thrombocytopenic mice lacking transcription factor NF-E2, we followed the fate of radiolabeled Tpo administered in vivo. In contrast to control mice, in which a significant fraction of the radioligand associates with platelets in the cellular fraction of whole blood, only a background level of radioactivity is detected in the same fraction from NF-E2 knockout mice. Additionally, a much lower level of radioactivity is associated with the lungs of these mutant mice com-
pared with controls, and we find little $^{125}$I-Tpo in the plasma. In hematopoietic tissues, the radioligand binds directly to megakaryocytes from normal or mutant animals, consistent with a role for these Mpl-expressing cells in modulating free Tpo levels, as previously suggested.\textsuperscript{11,13,15}

Surprisingly, in the spleen of mutant mice, we also note binding of $^{125}$I-Tpo to small, previously undetected particles that resemble megakaryocyte fragments or poorly developed platelets and are usually present within or in close association with macrophages. These particles can thus sequester free Tpo from the serum and, together with the abundant megakaryocytes, likely contribute to the paradoxically low concentration of free Tpo in the serum of NF-E2 knockout mice. Although the precise nature of these structures remains to be elucidated, they are not present in the blood in appreciable numbers, as judged by the very small amount of immunoreactive c-Mpl protein in the insoluble plasma fraction and our inability to detect them visually in the blood. Rather, the sum of our observations suggests that in vivo these particles are recognized as being abnormal and are likely cleared rapidly by splenic macrophages.

Although the unique model of thrombocytopenia in mice lacking transcription factor NF-E2 provides a useful molecular inroad to megakaryocyte development and platelet biogenesis, it also exposes some apparent paradoxes. First, it reveals an apparent concordance rather than the usual inverse correlation between platelet numbers and serum Tpo levels. Second, it raises the question of alternate hemostatic mechanisms that permit survival, albeit with chronic bleeding, in a small fraction of mutant mice. Our present findings address each of these issues. We have shown that free Tpo is cleared in these knockout mice by cells in the bone marrow and spleen, including megakaryocytes and small, platelet-like particles or megakaryocyte fragments. The latter structures, which in turn appear to be cleared rapidly by macrophages, likely also provide some degree of hemostasis to avoid hem-
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orraghic death in a few mutant mice. Further studies should clarify the nature of these particles and also provide a grasp of the molecular processes that prevent normal thrombocyto-

poiesis in the absence of NF-E2.

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