

Thrombopoietin: From Theory to Reality

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The term thrombopoietin (TPO) was first penned in 1958 to describe the primary regulator of platelet production. Multiple efforts to purify the hormone throughout the 1970s and 1980s were unsuccessful, because of the scarcity of the protein in even the richest physiologic source of the hormone (thrombocytopenic plasma), and the biochemical complexity of this as a starting material for purification^(1,2). However, the cloning of the proto-oncogene *c-mpl*⁽³⁾ opened an alternative route to the identification of TPO. Detailed study of the *c-mpl* gene revealed it to encode an orphan hematopoietic cytokine receptor, and its tissue distribution and experiments designed to eliminate its expression suggested that *c-mpl* encodes the TPO receptor^(4,5). Using three distinct strategies, five separate groups either purified protein or cloned cDNA for TPO in 1994⁽⁶⁾. This review will focus on the physiology of TPO production and function, concentrating on recent findings that help to explain platelet homeostasis and how our understanding of TPO biology can translate into better care of thrombocytopenic patients.

The Molecular Biology of Thrombopoietin

The cloned human TPO cDNA encodes a predicted polypeptide of 353 amino acids, including a 21 amino acid secretory leader sequence⁽⁷⁻¹⁰⁾. Like all of the other known hematopoietic growth factors⁽¹¹⁾, application of secondary structure algorithms indicate that TPO folds into a four alpha helix bundle, with two long loops connecting the first and second, and third and fourth helices. Several of the amino acids on the first, third and fourth helices and two of the interhelical loops that interact with the Mpl receptor have also been mapped^(12,13). The TPO gene localizes to human chromosome 3q21⁽¹⁴⁾, a region rearranged in a patient with thrombocytosis and blastic transformation of chronic myeloid leukemia⁽¹⁵⁾. The polypeptide can be conceptually divided into two domains; the amino-terminal 154 residues of the mature polypeptide bears striking sequence homology with erythropoietin (EPO) and binds to the Mpl receptor. The carboxyl-terminal domain bears no resemblance to any known proteins, but contains multiple sites of both N- and O-linked carbohydrate modification (**Figure 1**). This latter feature accounts for the large discrepancy between the predicted and actual M_r of the protein; 50% of the 70 kDa TPO molecule is carbohydrate. Functions for the carbohydrate containing carboxyl-terminal domain include increasing the circulatory survival of the protein⁽¹⁶⁾, allowing for a tenfold increase in bioavailability of the full length form over one lacking the carboxyl-terminal region, and improving the secretory efficiency of TPO. Here, again, inclusion of the carboxyl-terminus of the protein enhances secretion of TPO tenfold over that seen in

cells expressing the truncated protein⁽¹⁷⁾. The molecular basis for this finding is uncertain, but the two most intriguing models propose that the carboxyl-terminus of the protein aids in folding of the receptor binding domain, or that it protects the amino-terminus from proteolytic destruction during its intracellular processing. Studies in multiple laboratories are underway to better understand the implications of these findings both for TPO in particular, and for the physiology of protein secretion in general.

The Physiology of Thrombopoietin

The availability of recombinant thrombopoietin has allowed the rapid physiologic characterization of the protein. The hormone is the major regulator of megakaryocyte maturation, supporting 1) the formation of platelet-specific granules; 2) demarcation membranes and platelet fields; 3) the expression of platelet specific membrane proteins, including the glycoprotein (gp) IIb/IIIa fibrinogen receptor and the gp Ib/V/IX von Willebrand factor receptor; 4) megakaryocyte adhesion through activation of gpIIb/IIIa, VLA-4 and VLA-5; 5) endomitosis and its resultant polyploid state; and 6) the formation of platelets from single megakaryocytes in serum-free culture⁽¹⁸⁻²²⁾. Other cytokines fail to induce similar levels of megakaryocyte maturation if endogenous TPO effects are blocked⁽¹⁸⁾. The hormone is also a very potent inducer of proliferation of megakaryocytic progenitor cells. By itself, optimal levels of the hormone can induce the proliferation of up to 75% of all marrow progenitor cells committed to the megakaryocyte lineage^(20,23), and at lower levels the hormone acts synergistically to enhance megakaryocyte development in combination with interleukin (IL)-3, stem cell factor (SCF), IL-11 and EPO⁽²⁴⁾. At least one of the mechanisms by which TPO acts is by suppression of progenitor cell apoptosis⁽²⁵⁾.

In addition to its effects on megakaryocytes and their immediate precursors, TPO can support the survival of hematopoietic stem cells and acts in synergy with IL-3 and SCF to induce these cells into the cell cycle and increase their output of both primitive and committed hematopoietic progenitor cells of all lineages^(26,27). These *in vitro* effects are reflective of *in vivo* events; genetic elimination of TPO or its receptor is associated with reduction of the numbers of marrow hematopoietic progenitor cells of all lineages to approximately 25% of normal values^(28,29), the number of primitive spleen colony-forming cells⁽³⁰⁾, and the number of long-term repopulating hematopoietic stem cells⁽³¹⁾.

The Regulation of Thrombopoietin Production

The preponderance of available data indicates that TPO blood levels vary inversely with the combined megakaryo-

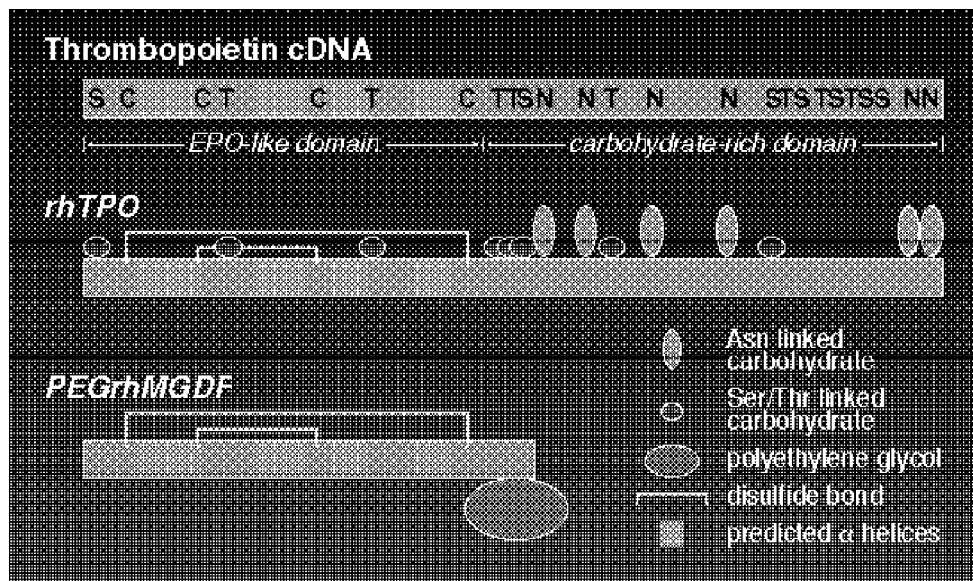


Figure 1. Thrombopoietin and its relationship with Erythropoietin. The polypeptide structure of TPO is illustrated and the features shared with EPO highlighted, including four alpha helices, multiple sites of both N- and O-linked carbohydrate modification, and a carboxyl-terminal extension which bears no similarity to any known proteins.

cyte and platelet mass. The explanation for the inverse relationship is thought to lie in the constitutive production of the hormone in several organs (e.g. liver and kidney⁽³²⁾) and the presence of Mpl receptors on megakaryocytes and platelets. Most investigators believe the relatively fixed total body TPO production rate is moderated by plasma uptake and destruction by cells bearing Mpl receptors⁽³³⁾. Thus, when platelet numbers are high, most of the thrombopoietin produced is destroyed, leaving little to stimulate thrombopoiesis. In contrast, in the presence of thrombocytopenia, particularly those states with concomitant megakaryocyte deficiency (aplastic anemia, myelosuppressive therapy), the plasma concentration of thrombopoietin is high because of low levels of Mpl-mediated destruction. Although it is clear that this model can account for some aspects of thrombopoietin availability, careful examination of the available data indicates that additional mechanisms must contribute to the regulation of hormone production during times of increased platelet demand. For example, a precise indirect relationship between thrombopoietin levels and platelet count does not hold in several thrombocytopenic states, such as immune thrombocytopenic purpura⁽³⁴⁾, and even in thrombocytopenic patients with high TPO levels there is no strict relationship between the two parameters⁽³⁵⁾. In cases of platelet destruction, in which megakaryocytic hyperplasia occurs, it has been suggested that the increased megakaryocyte mass is responsible for increased hormone catabolism, masking what would otherwise have been an increased thrombopoietin state⁽³⁶⁾. However, if this were strictly the case, an inverse relationship between megakaryocyte volume and thrombopoietin levels should exist, a prediction not supported by the one study in which these two

parameters were carefully assessed⁽³⁷⁾. It is also possible that the reduced levels of thrombopoietin in some states of platelet destruction is *responsible* for the thrombocytopenia per se. For instance, about one quarter of patients with what is otherwise typical idiopathic thrombocytopenic purpura do not display increased platelet turnover or megakaryocytic hyperplasia⁽³⁸⁾. Thrombopoietin levels have not been checked in this subset of patients, but reduced levels (by either immune targeting or other mechanisms) could account for the thrombocytopenia found in these patients. Moreover, additional mechanisms for thrombopoietin regulation have been identified. For example, interferon- γ and TNF- α upregulate hepatic endothelial cell thrombopoietin mRNA⁽³⁹⁾ and IL-11 upregulates hormone production from marrow accessory cells⁽⁴⁰⁾. These findings could help to explain why some patients with reactive thrombocytosis display blood thrombopoietin levels too high for their platelet count⁽⁴¹⁾. And thrombopoietin production from liver cells falls in the presence of interferon- α , potentially helping to explain the worsening of thrombocytopenia associated with the use of that agent for viral liver disease⁽⁴²⁾, at least in part.

The Thrombopoietin Receptor, c-Mpl

The thrombopoietin receptor was initially cloned as the cellular homologue of a murine transforming oncogene, *v-mpl*⁽³⁾. Recognized as a hematopoietic growth factor receptor by virtue of its characteristically spaced cysteine residues and tryptophan-serine-anything-tryptophan-serine juxtamembrane pentapeptide (the "WSXWS box"), several features of *c-mpl* expression suggested an important role in megakaryocyte formation^(3,4). This supposition was confirmed with the cloning of thrombopoietin. Equilibrium

binding experiments with radiolabeled thrombopoietin reveal a single class of receptors with a binding affinity of ~100 pM; platelets display approximately 25 receptors per cell⁽⁴³⁾. Megakaryocytes display far greater numbers of receptors, although their precise quantitation has been technically challenging.

In addition to the signaling isoform of Mpl, (termed the P form for a unique Pst I restriction endonuclease site present in the region encoding the cytoplasmic domain), several other isoforms of the receptor have been predicted to exist based on the presence of alternate forms of mRNA identified in receptor bearing cells. Potential alternate isoforms of Mpl include 1) a soluble version of Mpl (which arises by splicing out of the exon encoding the WSXWS and transmembrane regions⁽⁵¹⁾); 2) a membrane bound form of the receptor (the K form, for a unique Kpn I site) derived from retention of the tenth Mpl intron, and encoding a polypeptide that lacks the carboxyl terminal 113 residues (eliminating the box1/box2 and most of the other signaling motifs recognized in the intracytoplasmic domain of the receptor); and 3) another membrane-bound form that lacks 24 amino acids of the extracellular domain. It is unlikely that any of the alternate forms of Mpl other than the K form of Mpl bind thrombopoietin (e.g. mutation of the WSXWS region of the growth hormone receptor unfolds the proteins⁽⁴⁴⁾) and none likely signals, potentially making their presence splicing artifacts. It is formally possible, however, that the K form of Mpl acts as a decoy receptor.

The binding of thrombopoietin to Mpl is believed to result in receptor dimerization⁽⁴⁵⁾, an event that leads to the cross-phosphorylation and activation of members of the Janus family of kinases. JAK activation appears to be a critical event for signaling; genetic elimination of JAK2 leads to fetal lethality due to a failure of hematopoiesis⁽⁴⁶⁾. Like several other hematopoietic growth factor receptors, the binding of thrombopoietin to Mpl induces JAK2 and TYK2 activation (although the latter is of less importance [Drachman JG, Millett KM, Kaushansky K: Mpl signal transduction requires functional JAK2, not TYK2. *J Biol Chem*, *in press*]), which then phosphorylate a subset of receptor Tyr residues, forming docking sites for several signaling intermediates. Proteins bound and subsequently activated by JAK2 include the nascent transcription factors STAT3 and STAT5, the adapter proteins Grb2, Shc and its associated phosphatase SHIP, the GTP exchange factors Vav and SOS, and the hematopoietic receptor-related phosphatase SHP-2⁽⁴⁷⁻⁵²⁾. However, two important questions have recently arisen in the field. First, is JAK activation sufficient to induce all of the effects of Mpl receptor engagement, and second, are the tyrosine residues of the receptor critical or superfluous targets of JAK2? Several recent experiments have shed surprising new insights on these questions.

Evidence from several quarters suggests that JAK2 activation is sufficient to transmit a proliferative stimulus in hematopoietic cells. For example, the introduction of a fusion receptor composed of the extracellular and transmem-

brane domains of the erythropoietin receptor and the kinase domain of JAK2 induces cellular proliferation in hematopoietic factor dependent FDC2 cells⁽⁵³⁾. A similar result was reported for an epidermal growth factor (EGF) receptor/JAK2 fusion in a similar hematopoietic cell line, 32D⁽⁵⁴⁾. In contrast, we have found that a similar strategy linking the thrombopoietin receptor to JAK2 fails to support the proliferation of yet another growth factor dependent cell line, BaF3 cells, indicating the danger in relying too heavily on the results from transformed cell lines. Moreover, none of these cell types differentiates into normal blood cells, precluding any conclusions of the role of JAK2 in cellular differentiation. Only from the study of receptor mutants introduced into primary cells can one be certain whether JAK activation is sufficient for receptor signaling, studies that are actively underway in a number of laboratories.

The second question of whether the tyrosine residues that become phosphorylated in response to thrombopoietin binding are critical for receptor signaling has also yielded an unexpected answer. Introduction of an Mpl receptor that has been truncated 69 residues beyond the transmembrane domain was introduced into CD41-selected, gpIIb-expressing marrow cells derived from an *mpl*^{-/-} mouse. Cells were then grown in the presence of thrombopoietin and evaluated. We found that an equal number of megakaryocytes, of full maturity, developed in response to thrombopoietin as when the full length *mpl* receptor was used. As the truncated Mpl receptor is not phosphorylated on tyrosine in response to thrombopoietin, at least in BaF3 cells, these results suggest that the phosphotyrosine docking sites induced by hormone binding are not essential for receptor function. A similar conclusion was derived from studies using homologous recombination to introduce the same truncated receptor into an *mpl*^{-/-} mouse⁽⁵⁵⁾. These results stand in contrast to those obtained by eliminating all of the tyrosine residues of the erythropoietin receptor⁽⁵⁶⁾. In these studies, alteration of the eight erythropoietin receptor cytoplasmic domain tyrosine residues to phenylalanine abrogated the capacity of the receptor to transduce a differentiative signal in fetal liver erythroid progenitor cells. Thus, it appears that the signals derived from all receptors are not qualitatively similar.

Other hematopoietic growth factors also activate members of the Src family of cytoplasmic kinases, an event required for cellular proliferation⁽⁵⁷⁾. Many of these molecules (e.g. SOS⁽⁵⁸⁾ or Src⁽⁵⁹⁾) activate the Ras/Raf and PI3K pathways, which have profound effects on mediators of cell cycle control and survival. For example, GTP-Ras activates Raf-1⁽⁶⁰⁾, which then activates 1) the *cdc25A* phosphatase⁽⁶¹⁾, critical for early cell cycle progression; 2) MEK-1, essential for mitogen-activated protein kinase (MAPK) activation (see below), and when translocated to mitochondria; and 3) the *bcl* family member *bad*, preventing its interaction with and neutralization of the cell survival factor *bcl-2*⁽⁶²⁾. PI3K activates Akt, also a critical mediator of cell survival.

MAPKs act primarily upon nuclear transcription factors, including members of the Ets, Rel and AP-1 families⁽⁶⁰⁾. The genes affected by these transcription factors play important roles in cell proliferation and survival, and include p21, p27, G₁ cyclins, bcl family members, and caspase inhibitors. MAPKs are also thought to play an important role in megakaryocyte physiology, as prolonged MAPK activation was linked to differentiation in the UT-7/mpl cell line⁽⁶³⁾. More recently, we have had the opportunity to test the effects of MAPK blockade in primary megakaryocytes, using PD 98059, an inhibitor of the MAPK activating enzyme MEK-1. We found that the inhibitor reduced the number of megakaryocytes present following a four day culture in thrombopoietin, and substantially blocked their polyploidization (Rojnuckarin P, Drachman JG, Kaushansky K: Thrombopoietin-induced activation of the mitogen-activated protein kinase pathway in normal megakaryocytes: Role in endomitosis. Submitted for publication).

Thrombopoietin and Clinical Hematology

Finally, the role of recombinant thrombopoietin as a therapeutic agent has been evaluated in several settings. The toxicity and therapeutic efficacy of thrombopoietin has been tested in mice, rats, dogs, non-human primates, cancer patients and normal volunteers. In all studies administration of a single dose of any form of thrombopoietin was safe; specifically, there were no signs of hepatic, renal or pulmonary toxicity, capillary leak, coagulopathy or hematopoietic toxicity, and no evidence of the acute phase response characteristic of many of the other cytokines known to affect megakaryocyte development. However, when a modified version of thrombopoietin, termed PEGylated recombinant human megakaryocyte growth and development factor (PEGrhMGDF), was given subcutaneously on multiple occasions to normal platelet pheresis volunteers, approximately 1% of individuals developed antibodies that appeared to cross-react with their own thrombopoietin, resulting in prolonged thrombocytopenia. Such complications have not been seen in cancer patients given the drug. Nevertheless, this result lead to the discontinuation of trials of the agent in normal platelet transfusion donors.

When administered to animals treated with modest doses of either chemotherapy, radiation, or both, thrombopoietin has consistently resulted in higher nadir platelet counts and accelerated platelet recovery⁽⁶⁴⁻⁶⁷⁾. It also has displayed favorable effects on erythrocyte and granulocyte recovery in animal models, consistent with its *in vitro* stimulatory effects on cells of these hematopoietic lineages^(68,69). In one study conducted in rhesus monkeys, administration of thrombopoietin after sublethal irradiation resulted in functional iron deficiency, confirming its effect on erythropoiesis and arguing for study of prophylactic administration of iron in patients receiving thrombopoietin⁽⁶⁹⁾. Together, these results suggest that the therapeutic efficacy of the hormone might be broader than initially anticipated.

In contrast to the successful preclinical trials of the hormone in ameliorating thrombocytopenia and often pancy-

topenia associated with non-ablative myelosuppressive therapies, the effectiveness of thrombopoietin in accelerating platelet recovery following stem cell transplantation has been less than impressive. The administration of various forms of the hormone to lethally irradiated mice receiving marrow cells accelerated platelet recovery by two to four days^(70,71), but did not augment hematopoietic recovery when lower numbers of bone marrow cells were given⁽⁷²⁾. In mice receiving peripheral blood stem cell transplants the hormone was less effective⁽⁷³⁾.

Another potential use for thrombopoietin is to improve the quality of the marrow or stem cells to be used for transplantation. In one study designed to address this issue, thrombopoietin was administered to murine marrow donors, and the hematopoietic recovery of the lethally irradiated recipients monitored⁽⁷²⁾. Compared with mice receiving marrow from normal donors, both platelet and red cell recovery was more rapid in animals receiving marrow cells from thrombopoietin-treated mice, irrespective of whether the recipients received the hormone or not. The results were similar in a study of peripheral blood stem cell transplant in mice⁽⁷³⁾. However, given the adverse effects of PEGrhMGDF on normal platelet transfusion donors, the role of this agent in mobilizing or preparing stem cell donors is uncertain.

The results of several thrombopoietin toxicity trials in cancer patients are now available⁽⁷⁴⁻⁷⁶⁾. In all studies the administration of either recombinant human thrombopoietin (rhTPO) or PEGrhMGDF was safe, and when administered prior to chemotherapy was associated with substantial increases in platelet production. Although no signs of local or systemic toxicity were observed in these studies, it must be noted that patients with a history of cardiac, pulmonary, vascular or thrombotic disease were excluded from all phase I/II clinical trials.

Results from three trials of rhTPO or PEGrhMGDF in a total of 101 patients with cancer treated with carboplatin-based chemotherapeutic regimens have been reported.⁽⁷⁵⁻⁷⁷⁾ In all three studies platelet counts returned to baseline significantly faster, and in two of the three studies nadir platelet counts were higher in the patients given recombinant hormone, as compared with either those given placebo or the same patients during their first cycle of chemotherapy. However, the chemotherapy regimens administered in two of these trials induced only modest thrombocytopenia (mean nadir platelet counts in the placebo groups of 111,000/ μ l⁽⁷⁵⁾ and 60,000/ μ l⁽⁷⁷⁾); in neither study was the hospital course shortened or were significant numbers of platelet transfusions required. In the third study⁽⁷⁶⁾, compared to a first cycle of chemotherapy without rhTPO, nadir platelet counts and the number of thrombocytopenic days were both significantly improved by the use of the hormone following the second cycle of chemotherapy ($p < 0.001$). Moreover, of the patients enrolled in this study, 59% required platelet transfusions after their first cycle of chemotherapy, but only 26% needed them following the cycle of chemotherapy augmented with rhTPO ($p = 0.02$).

Thrombopoietin increases the number of megakaryo-

cytic and other hematopoietic progenitors *in vitro* and *in vivo*, and it mobilizes stem and progenitor cells from the marrow into the circulation, properties that might benefit patients receiving stem-cell transplants. In 40 patients with breast cancer undergoing autologous bone marrow transplantation⁽⁷⁸⁾, administration of PEGrhMGDF led to a 5 to 6 day earlier rise in platelet count to 20,000/ μ l and a 48 percent reduction in the use of platelet transfusions as compared with placebo treated patients. However, it has not been effective in patients undergoing autologous peripheral blood stem cell transplantation^(79,80).

Thrombopoietin has moved from concept to reality in the past five years. Its physiological properties have been extensively studied; the results have both supported old concepts and generated new ones, but have clearly established that the hormone is the primary regulator of megakaryocyte and platelet production. Despite these advances, many exciting new findings lay ahead. Virtually nothing is known of the genes responsible for commitment to the megakaryocytic lineage, elimination of the normal control mechanisms that allow polyploidy to develop, or the molecular mechanisms responsible for platelet formation. The availability of the primary regulator of megakaryocyte and platelet formation should provide investigators with important new tools to probe these fascinating questions, and may also provide a means to reduce the toxicity of marrow failure.

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