Determinants of platelet number and regulation of thrombopoiesis

Kenneth Kaushansky

Department of Medicine, University of California, San Diego, CA

Our understanding of thrombopoiesis has improved greatly in the last two decades with the availability of in vitro assays of megakaryocyte progenitor cell growth, with the cloning and characterization of stem cell factor (SCF) and thrombopoietin (Tpo), the latter the primary humoral regulator of this process, and with the generation of genetically altered murine models of thrombopoietic failure and excess. While SCF affects developmentally early aspects of megakaryocyte growth, Tpo affects nearly all aspects of platelet production, from hematopoietic stem cell (HSC) self-renewal and expansion, through stimulation of megakaryocyte progenitor cell proliferation, to supporting their maturation into platelet-producing cells. The molecular and cellular mechanisms through which the marrow microenvironment and humoral mediators affect platelet production provide new insights into the interplay between intrinsic and extrinsic influences on hematopoiesis, and highlight new opportunities to translate basic biology into clinical advances.

Overview of Platelets and Thrombopoiesis

A n adequate supply of platelets is essential to repair the minute vascular damage that continuously occurs and to initiate thrombus formation in the event of overt vascular injury. Platelets also play vital roles in wound repair, the innate immune response and metastatic tumor cell biology. The average platelet count in humans ranges from 150 to $350 \times 10^9$, although the level for any individual is maintained within fairly narrow limits from day to day. While this “normal range” is derived from the mean $\pm 2$ SD of a group of “healthy” individuals, epidemiological evidence indicates that individuals who display a platelet count in the highest quartile of this range have a 2-fold increased risk of adverse cardiovascular events, and in both experimental animal models of metastatic cancer and in patients with tumors higher platelet levels carry an unfavorable prognosis.

With a lifespan of $\sim 10$ days, a blood volume of 5 L, and one third of platelets pooled in the spleen, each day the average adult human must produce $\sim 1 \times 10^{11}$ platelets to maintain a normal platelet count under steady-state conditions, a level of production that can greatly increase under conditions of increased demand. Thrombopoiesis is dependent on the marrow microenvironment, composed of both cells and extracellular matrix proteins, and cell surface and soluble hematopoietic growth factors. Of the cellular microenvironmental components, endothelial cells appear to play the biggest direct role, as judged by the close juxtaposition of mature megakaryocytes and sinusoidal endothelial cells, and interstitial marrow stromal cells, which elaborate Tpo in response to thrombocytopenia. Of the extracellular matrix components, fibronectin engagement of integrin $\alpha_4\beta_1$ stimulates megakaryopoiesis, and vitronectin engagement of integrin $\alpha V \beta 3$ enhances platelet formation. And while a large number of hematopoietic growth factors have been shown to affect megakaryocyte growth or maturation, including interleukin (IL)-3, granulocyte-macrophage colony-stimulating factor, IL-6, IL-11, leukemia inhibitory factor, SCF and Tpo, all but the latter two are dispensable for normal and stimulated thrombopoiesis in vivo. For example, mice with severely reduced levels of SCF (Skit/Skit) display (in addition to severe anemia) thrombocytopenia. Likewise, genetic elimination of Tpo or the Tpo receptor, c-Mpl, reduces basal platelet levels to 10% to 20% of normal. These animal proof-of-principle experiments have a counterpart in human disease; congenital amegakaryocytic thrombocytopenia (CAMT; see below), a pediatric condition in which neonates display severe thrombocytopenia and later develop aplastic anemia as HSC numbers deteriorate, in nearly all cases is due to genetic loss of function of the c-Mpl gene. The production of SCF and Tpo is ubiquitous; virtually every tissue elaborates SCF, and its blood levels do not vary with any blood cell counts. In contrast, while widespread in production, blood levels of Tpo vary widely, in an inverse relationship to platelet count. Our understanding of Tpo production is most
advanced for hepatic and marrow stromal cell production of the hormone.

Quantitatively, the liver is the richest source of Tpo production; reciprocal liver transplant experiments between normal and Tpo-null mice established that roughly half of all Tpo at steady state is produced in the liver.6 Moreover, patients with hepatic failure display clinically significant thrombocytopenia, due in large measure to reduced levels of Tpo production, which is rapidly reversed by liver transplantation.9 Hepatic production of Tpo is largely constitutive, with mRNA and protein levels unresponsive to alterations in platelet count.10 In contrast, in patients with reactive thrombocytosis secondary to inflammation, IL-6 is responsible for enhanced hepatic production of Tpo11,12 and enhanced thrombopoiesis.

While the importance of hepatic Tpo is clear, marrow stromal cell production of the hormone is less well established. However, multiple lines of evidence have established that the marrow stroma can produce Tpo, and that thrombocytopenia induces a striking increase in Tpo mRNA and protein.13-14 Moreover, the molecular mechanisms that regulate this process have been traced to platelet granule proteins, which serve to inhibit stromal cell transcription of the Tpo gene.13-14 The sites in the Tpo gene responsible for its transcriptional regulation are beginning to be discerned.14 In addition, it is clear that Tpo expression can be affected by alterations in the efficiency of its mRNA translation into protein, at least under pathological circumstances.

The initiation codon at which Tpo is translated has been mapped and is the 8th ATG codon in the 5’ untranslated region of the Tpo mRNA.13 Of great importance, the 8th ATG is embedded in a short open reading frame initiated by the 7th ATG. As such, translation efficiency of Tpo is very poor. A number of mutations of this region of the Tpo have been described in a series of families with familial thrombocytosis, in which translation efficiency is greatly improved, leading to enhanced Tpo levels and thrombocytosis (see below).

In addition to Tpo, it is likely that additional factors influence thrombopoiesis, as the genetic elimination of the hormone or its receptor in mouse or man leads to profound but not absolute thrombocytopenia (the platelet counts in these settings is ~10% of a normal level). To determine whether any other growth factors contribute to thrombopoiesis in the c-Mpl null state, these mice were crossed with other cytokine- or cytokine receptor–deficient animals; from these studies it is clear that IL-3, IL-6, IL-11 and LIF17 are not basal, physiological mediators of thrombopoiesis. However, the chemokine stromal cell–derived factor (SDF)-1 (also termed CXCL12) exerts numerous influences on megakaryopoiesis, and recent studies indicate it may be responsible for thrombopoiesis not related to thrombopoietin. For example, SDF-1 acts alone and in synergy with Tpo to enhance megakaryocyte colony formation in serum-free culture.18 The chemokine also affects megakaryocyte motility, driving their migration towards stromal cells,19 with which they productively interact in an integrin α4β1-dependent manner. The administration of SDF-1, along with fibroblast growth factor (FGF)-4, can nearly normalize the platelet count of c-Mpl–deficient mice and enhances platelet recovery following myelosuppression.20 Thus, SDF-1 and marrow stromal cells exert important influences upon thrombopoiesis, but whether levels of the chemokine or surface expression of integrins can be modulated in response to thrombocytopoiesia remains uncertain.

### Mpl Receptor Expression, Regulation and Signaling

The type I hematopoietic cytokine receptor family, of which c-Mpl is a member, consists of more than 20 molecules that bear one or two cytokine receptor motifs (CRMs), an ~200 amino acid module containing four, spatially conserved Cys residues, 14 β-sheets, and a juxtamembrane Trp-Ser-Xaa-Trp-Ser sequence. In addition to the CRM(s), type I receptors contain a 20-25 residue transmembrane domain and a 70-500 amino acid intracellular domain containing short sequences that bind intracellular kinases and other signal transducing molecules. c-Mpl is expressed primarily in hematopoietic tissues, specifically in megakaryocytes, their precursors and their progeny.21 For the most part, the receptor is constitutively expressed in these tissues, although its display is modulated by Tpo binding and receptor internalization, after which c-Mpl is either recycled to the cell membrane or destroyed by both proteasomal and lysosomal pathways.22

The c-Mpl gene contains 12 exons and is organized like other members of the hematopoietic cytokine receptor family.23 Of note, expression levels of c-Mpl are low, with only 25 to 100 surface receptors present per platelet.24 Properly regulated expression of c-Mpl is critical for controlling thrombopoiesis, as expression of c-Mpl from a truncated promoter leads to excessive thrombocytosis, likely due to greater expression in megakaryocyte progenitors, where it drives cell proliferation, and lesser expression in platelets, where it acts to reduce blood levels of thrombopoietin by adsorption.25 The origin of the poor expression of c-Mpl appears to be related to the c-Mpl-tr isoform, as its co-expression with full length c-Mpl leads to rapid degradation of the latter.25 However, whether this physiology is reflected in Tpo signal regulation is, at best, only speculative at present.
Another aspect of c-Mpl regulation under intense study is its expression on blood cells of patients with myeloproliferative disorders (MPDs). While easily detectable on normal marrow megakaryocytes and blood platelets, the receptor is down-modulated on platelets from patients with polycythemia vera and essential thrombocytopenia. While the molecular basis for this observation is not yet understood, it could be related to the hypersensitivity to cytokines and signaling abnormalities seen in these disorders. A growing body of evidence links Tpo/c-Mpl to the MPDs, including in vitro data indicating that Jak2V617F, the most common acquired genetic mutation found in patients with MPDs, must bind to a homodimeric cytokine receptor in order to impart growth factor hypersensitivity to cells in culture. As c-Mpl is the only hematopoietic cytokine receptor expressed in HSCs, the cell of origin of the MPDs, it is likely from these arguments that c-Mpl is involved in some way in the MPDs.

Upon binding cognate ligand hematopoietic cytokine receptors such as c-Mpl are activated to transmit numerous biochemical signals. The membrane proximal box1 and box2 cytoplasmic domains of the receptors constitutively bind JAK family kinases, even in an inactive state. Upon ligand binding, the induced conformational shift results in cross-phosphorylation and activation of the two tethered Jak2 kinases, initiating signal transduction. The active JAK kinase then phosphorylates (1) tyrosine residues within the cytoplasmic domain of the receptor itself, (2) molecules that dock to the receptor P-Tyr residues, molecules that for the most part promote cell survival and proliferation, including the signal transducers and activators of transcription (STATs), phosphoinositol-3-kinase (PI3K) and the mitogen-activated protein kinases (MAPKs), and (3) after some delay, activate or bind additional molecules that limit cell signaling, including suppressors of cytokine signaling (SOCS) and the SHP1 and SHIP1 phosphatases.

Additional insights into how the JAK kinases are regulated have come from domain analysis of the proteins. All four members of the family (JAK1, JAK2, JAK3 and Tyk2) display three major domains, JH (Jak homology)1, JH2 and FERM (Four-point-one, Ezrin, Radixin, Moesin), the latter responsible for binding to the cytoplasmic domain of the cytokine receptors. The JH1 domain carries the kinase activity of JAKs, and while JH2 bears significant homology to JH1, its active site is altered and inactivated, and is thus termed the pseudokinase domain. The function of JH2 was identified by differential expression studies; the JH1 domain is an active kinase when expressed alone, whereas the activity of a JH1/JH2 polypeptide is greatly reduced. Of great interest, and discussed at some length below, is the activity of a JH1/JH2 polypeptide is greatly reduced.28

Of great interest, and discussed at some length below, is the activity of a JH1/JH2 polypeptide is greatly reduced.28

For the Tpo/c-Mpl system, the p85 regulatory subunit of PI3K is phosphorylated indirectly, and once paired with its p110 kinase subunit alters membrane phospholipids to induce the synthesis of phosphoinositols 3,4,5 phosphate, attracting plecstrin homology (PH) domain containing proteins to the membrane, the most well studied of which is Akt (also termed protein kinase B), where it is activated by PDK1-induced phosphorylation. Active Akt is a key mediator of Tpo-induced cell survival and proliferation in megakaryocytes and enhances α-granule secretion and aggregation in thrombin-stimulated platelets. Pathways that lie downstream of Akt in megakaryocytes and platelets are under study, and include the transcription factor FOXO3a, the cell cycle inhibitor p27, and glycogen synthase kinase (GSK) 3β. Phosphorylation of c-Mpl Tyr626 is a key event in Tpo signal transduction, as it serves as docking site for several adaptor molecules including Grb2 and Shc, which recruit SOS, ultimately leading to activation of Ras. Once Ras-GTP forms, a series of kinases is activated eventuating in phosphorylation and activation of two of the mitogen-activated protein kinase (MAPK) pathways, p42/p44 extracellular regulated kinase (ERK) 1 and 2, and p38 MAPK. The functional consequences for these events include induction of the transcription factor HoxB4 and expansion of HSCs mediated by p38 MAPK, translocation of the transcription factor HoxA9 from cytoplasm to nucleus, also favorably affecting HSC expansion, and the ERK1/2-induced proliferation and polyploidization of megakaryocytes.

Immature Megakaryopoiesis is Characterized by Polyploidy

Based upon its behavior in tissue culture, a typical megakaryocytic progenitor undergoes from 3 to 6 diploid cell cycles, giving rise to divisions to form from 8 to 64 progeny. At some point the usual diploid cell cycles are replaced by the endomitotic cell cycle, one of the most remarkable features of maturing marrow megakaryocytes. When complete, individual cells display 32, 64, 128 and even 256 times the normal chromosome complement in a single, highly lobated “polyploid” nucleus. As their name implies, megakaryocytes are extremely large cells (up to 300 mm in culture), which require massive protein synthesis capacity, supported by the simultaneous transcription of the multiple copies of each megakaryocyte-specific gene in a single cell. The molecular mechanisms that allow the cell to escape from the normally strict adherence to diploid cell cycle controls and display polyploidy are beginning to be understood. Careful analysis of videomicroscopic images of...
fluorescently labeled polyploid megakaryocytes demonstrates that mature megakaryocytes do not simply replicate their DNA and then skip mitosis (M), as was once assumed, but rather begin mitosis (M) phase, progress into late anaphase, begin to furrow and then abort the process, resuming a circular cell profile. Then, without having divided, begin G1 and S phase once again. This observation allows one to construct hypotheses as to the origins of the failure to complete anaphase and telophase, testing the proteins responsible for this particular phase of the cell cycle. We and others have found that mislocalization of the small G protein Rho is an attractive molecular candidate for the observed events in megakaryocyte endomitosis. It is clear that additional mechanisms are awaiting discovery; for example, it is completely unclear how the polyploid megakaryocyte escapes the programmed cell death that awaits normal cells that display additional chromosomes.

Mature Megakaryocytes Undergo Proplatelet Process Formation and Fragmentation
The final stage of thrombopoiesis is characterized by a massive rearrangement of the megakaryocyte cytoskeleton, in which long, branching cytoplasmic processes are extruded from the cell body, which then fragment into platelets at their termini. These “proplatelet processes” display remarkable motility and trafficking of the granules and proteins that characterize the mature platelet. As in polyploidy, videomicroscopic images have been vital for our understanding of this unique biological event. The structure of the proplatelet is dependent on β1 tubulin, which forms the skeleton and “highway” on which cargo of mitochondria and platelet specific granules traffic to the bulbous termini, where several spirals of tubulin form the mature platelet cytoskeleton. Actin dynamics are also important in this process, as the highly structured maturing megakaryocyte submembrane actin skeleton is disrupted, allowing the growing tubulin structures to “push” the cytoplasmic membrane into the highly elongated proplatelet process. While these concepts were developed using sophisticated freeze fracture and videomicroscopic imaging strategies in vitro, they have now been extended to living systems. Using open murine calvarial bone and emerging between sinusoidal endothelial cells fragmenting platelets in the hemodynamic stress of the marrow sinusoid. Despite these morphological advances, we remain unclear about what triggers proplatelet formation or its molecular underpinnings.

Future Research Directions
Our understanding of the molecular basis of thrombopoiesis has progressed substantially since James Homer Wright in 1906 provided evidence that megakaryocytes give rise to blood platelets. Until this time, little attention had been paid to platelets, then referred to as the “dust of the blood.” But despite great progress, many questions remain: What is the mechanistic basis for proplatelet formation? What is the reason for megakaryocyte polyploidy and what are the molecular mechanisms by which these cells uncouple DNA synthesis and cell division? What is being sensed in marrow stromal cells that alter their production of Tpo in thrombocytopenia? What is the role of c-Mpl in MPDs and how can an alteration in the Jak2 kinase lead to essential thrombocythemia in some patients and polycythemia in others? Research in thrombopoiesis is presently in a logarithmic growth phase, and with the availability of FDA-approved Tpo receptor agonists, the era of clinical application of our understanding of thrombopoiesis has now officially begun.

Disclosures
Conflict-of-interest disclosure: The author is a consultant for and serves on an advisory committee for Ligand Pharmaceuticals.
Off-label drug use: None disclosed.

Correspondence
Kenneth Kaushansky, MD, Chair, Department of Medicine, University of California, San Diego, 9500 Gilman Dr., Mailcode 0671, La Jolla, CA 92093-0671; Phone: 619-543-6170; Fax: 858-246-0223; e-mail: kkaushansky@ucsd.edu; Alternate e-mail: ttrettin@ucsd.edu

References
6. Ballmaier M, Gernshehausen M, Schulze H, et al. c-mpl mutations are the cause of congenital amegakaryocytic


