Megakaryocyte development and platelet production

Varda R. Deutsch¹ and Aaron Tomer²,³

¹The Haematology Institute, Tel Aviv Sourasky Medical Centre, Tel Aviv, ²Faculty of Health Sciences, Ben Gurion University of the Negev, and ³Soroka Medical Centre, Blood Bank and Transfusion Medicine, Beer-Sheva, Israel

Summary

Megakaryocytopoiesis involves the commitment of haematopoietic stem cells, and the proliferation, maturation and terminal differentiation of the megakaryocytic progenitors. Circulating levels of thrombopoietin (TPO), the primary growth-factor for the megakaryocyte (MK) lineage, induce concentration–dependent proliferation and maturation of MK progenitors by binding to the c-Mpl receptor and signalling induction. Decreased platelet turnover rates results in increased concentration of free TPO, enabling the compensatory response of marrow MKs to increased platelet production. C-Mpl activity is orchestrated by a complex cascade of signalling molecules that induces the action of specific transcription factors to drive MK proliferation and maturation. Mature MKs form proplatelet projections that are fragmented into circulating particles. Newly developed thrombopoietic agents operating via c-Mpl receptor may prove useful in supporting platelet production in thrombocytopenic state. Herein, we review the regulation of megakaryocytopoiesis and platelet production in normal and disease state, and the new approaches to thrombopoietic therapy.

Keywords: megakaryocytes, megakaryocytopoiesis, thrombopoietin, platelets, platelet production.

Megakaryocyte development

Megakaryocytes (MKS) give rise to circulating platelets (thrombocytes) through commitment of the multipotent stem cell to the MK lineage, proliferation of the progenitors and terminal differentiation of MKs. This process is characterised by DNA endoreduplication, cytoplasmic maturation and expansion, and release of cytoplasmic fragments as circulating platelets. Within the bone marrow (BM), MKs are derived from haematopoietic stem cells (HSCs), which evolve from the multipotential haemangioblast. The haemangioblast gives rise to all blood and blood vessel precursor cells. The HSC gives rise to the early common myeloid progenitor (CMP) that can be cloned as the multi-lineage (granulocyte, erythrocyte, MK and monocyte) colony-forming unit (CFU-GEMM). Erythroid and MK lineages arise from a common MK-erythroid progenitor (MEP) derived from the early CMP (Fig 1). CMP differentiation is orchestrated by molecular signals controlled by regulatory transcription factors (TF). Two major TF involved in CMP differentiation are GATA-1, which drives differentiation of MEP and PU.1, which regulates granulocyte-erythrocyte precursors (Nutt et al, 2005). The downregulation of PU.1 expression in the CMP is the first event associated with the restriction of differentiation to erythroid and MK lineages (Pang et al, 2005). In response to environmental factors, cytokines and chemokines, the bipotential MEP can develop into the highly proliferative, early MK burst-forming unit (BFU-MK), or the more mature smaller CFU-MK, which both express the CD34 antigen (Briddell et al, 1989). Alternatively, MEP can progress to early and late erythroid progenitors, the BFU-E and CFU-E (Schulze & Shivdasani, 2004). The proliferating diploid MK progenitors (megakaryoblasts) lose their capacity to divide, but retain their ability for DNA replication (endoreduplication) and cytoplasmic maturation.

Megakaryocyte maturation

The hallmarks of MK maturation are endoreduplication (polyploidisation) and expansion of cytoplasmic mass. Mature MKs give rise to circulating platelets by the acquisition of the cytoplasmic structural and functional characteristics necessary for platelet action (Patel et al, 2005; Richardson et al, 2005), reaching cell sizes <50–100 microns in diameter, with ploidy ranges up to 128 N (Tomer et al, 1987, 1988). Early megakaryoblasts have the highest nuclear/cytoplasmic ratio. These immature cells contain elevated RNA levels, prominent ribosomes and rough endoplasmic reticulum, express platelet peroxidase, contain α-granules and dense bodies, and primitive demarcation membrane. As the MK matures, the polyploid nucleus becomes horseshoe-shaped, the cytoplasm expands, and platelet organelles and the demarcation membrane system are amplified (Breton-Gorius & Reyes, 1976). The robust cytoplasmic mass forms proplatelet projections.
which give rise to de novo circulating platelets (Italiano et al., 1999; Italiano & Shivdasani, 2003). While the large polyploid MKs are easily identified, the small MK progenitor and immature MK are difficult to discern. Their identification is facilitated by using antibodies to major platelet membrane glycoproteins (GPs) including the integrin αIIbβ3 (CD41a or GPIIb/IIIa complex), CD41b (GPIIb), CD61 (GPIIIa), CD42a (GPIX), CD42b (GPIb) and CD51 (αV), as well as against the platelet α-granule proteins, von Willebrand factor (VWF), platelet factor 4 (PF4), β-thromboglobulin (β-TG), fibrinogen, coagulation factor VIII, and factor V (Tomer, 2004). Immature 2–4 N cells produce platelet peroxidase but have no detectable demarcation membrane or platelet α-granules (Breton-Gorius & Reyes, 1976). Anti-CD41 antibodies enable the detection of small ‘lymphoid’ megakaryoblasts in BM as well as CFU-MK by day 4 or 5 of culture (Mazur et al., 1981). Flow cytometry has also been adapted to the direct analysis of human MKs in routine BM aspirates (Tomer et al., 1988, 1989). Fluorescence activated cell sorting has enabled the separation of pure viable BM-derived MK populations (>98%) isolated on the basis of lineage markers. These cells were capable of responding to haematopoietic growth factor and synthesising both protein and DNA (Tomer et al., 1987). Moreover, flow cytometry has enabled the study of megakaryocytopenia in disease states, monitoring of drug effect in humans, and studying the effect of haematopoietic growth factors on megakaryocytopenia in humans and primates (Tomer & Harker, 1996; Harker et al., 1997; Tomer, 2002).

Recent flow cytometric studies have demonstrated that VWF is strongly expressed by early (2 and 4 N) marrow MKs, enabling their complete resolution from the other marrow cells at a level superior to that achieved with GPIIb/IIIa as a lineage-specific marker (Tomer, 2004).

Early MK differentiation is accompanied by the expression of GPIIX, which exists as a heterodimer complex with GPIb and GPV in the platelet membrane. In contrast, platelet GPIV and the thrombospondin (TSP) receptor (CD36) are later differentiation markers (Asch et al., 1987). Following activation, the platelet GPIIb/IIIa complex functions as a receptor for four adhesive proteins – fibrinogen, fibronectin, vitronectin and VWF, with GPIb being the major receptor for VWF (Tomer, 2004).

The nuclear endomitotic cell cycle consists of a DNA replication S-phase, an M-phase with multiple pole spindles, aborted anaphase B, aborted cytokinesis and a Gap-phase that enables re-entry into the next S-phase. Cyclin D3, is overexpressed in the G1-phase of maturing cells and is a key inducer of MK polyploidisation (Ravid et al., 2002). Cyclin E may also be important as cyclin E null mice have defective endomitosis of MK and trophoblasts (Geng et al., 2003). Aurora-B/AIM-1, the fundamental regulator of mitosis, has normal localisation and expression during prophase and early anaphase but is absent or mislocalised at late anaphase MK (Geddis & Kaushansky, 2004; Zhang et al., 2004). Chromosomes segregation is asymmetrical with normal metaphase/anaphase checkpoints (Roy et al., 2001). Ploidy classes are identified as

![Diagram](image-url)
geometric progressions of 2 N (diploid), i.e. 4, 8, 16 N, etc. The ploidy distribution obtained by flow cytometry in fractionated and unfractionated normal human marrow demonstrates 16 N for approximately half of the MK population, 23% of cells being 8 N or lower and 22% 32 N or higher (Tomer et al, 1989). The expression of GPIIb/IIa, GPIIIa, GP Ib and CD36 correlates directly with cell size and ploidy, as well as receptor density of GPIIb/IIa. The mean diameter of the MKs is 37 ± 4 μm (mean ± 1 SD), compared with 14 ± 2 μm of the total marrow cells, ranging from 21 ± 4 μm for 2 N cells to 56 ± 8 μm for 64 N cells (Tomer, 2004). The MK cytoplasmic mass may be calculated as the product of the mean MK cytoplasmic volume and the total number of MKs (Tomer, 2002). By determining the ratio of MKs to nucleated erythroid precursors in marrow preparations by flow cytometric means, the MK mass in patients with primary thrombocytosis was assessed (Tomer & Harker, 1996; Tomer, 2002). Following treatment with anagrelide, both cell frequency and volume decreased towards normal, resulting in decreased MK mass by about half, similar to the decrease in platelet count (Tomer, 2002). This finding of anagrelide effect was recently corroborated by in vitro studies (McCarty et al, 2006).

Platelet release occurs when the MK cytoplasm is transformed into proplatelets, followed by the release of 2000–5000 new platelets/cell (Long, 1998). Compensatory responses of marrow MKs increase platelet production through an increase in cell proliferation, maturation and release of platelets, as early as 24–48 h after inducing thrombocytopenia. Following thrombocytopenia and in thrombocytopenias of immune aetiology, the marked increase in MK number and size are notable (Mazur et al, 1988; Tomer et al, 1988). The normal frequency of human MK in BM is about one in 2000 nucleated cells. However, with compensatory response, the number increases 10-fold (Branchog et al, 1975), with an increased proportion of high-ploidy cells seen in BM aspirates (Tomer et al, 1989). Reciprocal decreases in MK size, ploidy and volume occur with experimentally induced thrombocytosis (Burstein et al, 1979; Jackson et al, 1984). These alterations, found in both experimental animals and human subjects, are primarily mediated by thrombopoietin (TPO) (Kaushansky, 2005a).

**Regulation of megakaryocytopoiesis**

The processes of megakaryocytopoiesis and platelet production occur within a complex BM microenvironment where chemokines, cytokines as well as adhesive interactions play a major role (Avecilla et al, 2004). Mechanisms regulating megakaryocytopoiesis operate at the levels of proliferation, differentiation and platelet release (Gewirtz, 1995; Kaushansky, 2003). In addition to the steady state megakaryocytopoiesis, which supplies 10^{11} platelets every day and a new turnover every 8–9 d, MKs also respond to changes in requirements for circulating platelets, increasing more than 10-fold under conditions that demand platelet production (Kaushansky, 2005a).

**Thrombopoietin**

Thrombopoietin, also known as c-Mpl ligand, is the primary physiological growth factor for the MK lineage, which also plays a central role in the survival and proliferation of HSC (Kaushansky, 2005b, 2006). TPO is the most potent cytokine for stimulating the proliferation and maturation of MK progenitor cells. It stimulates MKs to increase in cell size and ploidy, and to form proplatelet processes that then fragment into single platelets (Kaushansky, 2005a). In baboons, administration of recombinant human (rHu) TPO or polyethylene glycol derivative MK-growth-and-development-factor (PEG-rHuMGDF), produced a log-linear increase in marrow MK mass (cell number multiplied by volume) of up to 6.5-fold that was associated with marked increase in cell ploidy. Concordantly, there was increase in platelet count up to fivefold, reaching peak value after 2–4 weeks of injections (Fig 2). Because MK volume and ploidy attained predictable maximum values simultaneously, MK ploidy is an accurate measure of the Mpl-ligand stimulation of megakaryocytopoiesis (Tomer & Harker, 1996; Harker et al, 1997). TPO can also act in synergy with other haematopoietic cytokines and has been utilised effectively to expand human HSC and MK-progenitor cells in vitro (Pick et al, 2002; Bruno et al, 2003; Kaushansky, 2005b; Ivanovic et al, 2006).

In platelets, TPO enhances α-granule secretion and aggregation that is induced by thrombin in a phosphoinositide-3 kinase (PI3K)-dependent fashion (Kojima et al, 2001). This powerful cytokine also affects mature platelets, reducing the level of ADP, collagen, or thrombin necessary for aggregation (Oda et al, 1996; Pasquet et al, 2000), and stimulates platelet adhesion (Van Os et al, 2003).

Thrombopoietin is encoded by a single human gene, located on chromosome 3q26–3q27, which produces a 353 amino acid precursor protein. The mature molecule, composed of 332 amino acids, is acidic and heavily glycosylated. Interestingly, abnormalities (inversion or deletion) at its chromosomal locus are often found in megakaryocytic leukaemia and other myeloproliferative disorders associated with thrombocytosis (Yamamoto et al, 2000). Mutations in regulatory regions of the TPO gene, that result in overexpression of TPO and sustained intracellular signalling or disturbed regulation of circulating TPO, cause familial essential thrombocythaemia or familial thrombocytosis (Fig 3B) (Dame & Sutor, 2005; Kaushansky, 2005a). TPO shares high homology with erythropoietin (EPO) in its N-terminal half, reflecting a close evolutionary relationship between their receptor signalling pathways. TPO binds to its receptor on MKs and selectively initiates proliferation, maturation and cytoplasmic delivery of platelets into the circulation (Kaushansky, 1997, 2003). TPO is produced constitutively by the liver and its circulating levels are regulated by the extent of binding to c-Mpl receptors on
circulating platelets and marrow MKs resulting in the elimination of TPO-c-Mpl complexes (Kaushansky, 1997; Scheding et al., 2002). This can explain the delayed platelet recovery rate following chemotherapy and platelet transfusions (Slichter, 2004).

Messenger-RNA specific for TPO is also found in the kidney, marrow stroma and other tissues (Sungaran et al., 1997); however, its role in megakaryocytopoiesis is unclear. Blood and marrow levels of TPO are usually inversely related to BM MK mass and platelet counts (Kaushansky, 2005a). The plasma levels of TPO in normal state (95 ± 6 pg/l) increase several orders of magnitude in patients with aplastic anaemia or thrombocytopenia secondary to myelosuppression, and decline after platelet transusions or recovery of haematopoiesis (Kuter & Rosenberg, 1995; Kaushansky, 1997; Hsu et al., 1999) due to enhanced hepatic expression driven by the acute phase protein, interleukin 6 (IL-6) (Wolber et al., 2001). Mice in which either TPO or its ligand c-Mpl genes have been knocked out have severe thrombocytopenia with 10–20% residual platelet counts and small numbers of functionally normal MKs (Alexander et al., 1996). Their haematopoietic progenitors and their capacity to repopulate haematopoiesis in marrow-deficient mice are markedly reduced (Solberg, 2005). TPO, with its down-stream signalling pathways and diverse TFs is, therefore, central but not essential for megakaryocytopoiesis (Kirito & Kaushansky, 2006).

c-Mpl

The TPO receptor (c-Mpl) is predominantly expressed in haematopoietic tissues, haemangioblasts, MKs at all stages of differentiation and platelets. While receptor display is modulated by TPO binding and receptor internalisation, c-Mpl is usually constitutively expressed. TPO avidly binds and activates the c-Mpl receptors on MK and platelets which undergoes conformational changes to initiate signal transduc-
The Janus kinase (JAK) family kinases constitutively bind to the membrane-proximal cytoplasmic domains of c-Mpl and initiates signalling. The active JAK kinase also phosphorylates tyrosine residues within the receptor itself, as well as downstream signal transducers and activators of transcription (STATs), PI3K, and the mitogen-activated protein kinases (MAPKs). This cascade drives cell survival and proliferation, requires PI3K activation and activates SHP1 and SHIP1 phosphatases and suppressors of cytokine signalling (SOCSs) to limit cell signalling (Kaushansky, 2005b; Solberg, 2005).

Mice in which c-Mpl genes have been knocked out have severe thrombocytopenia, with residual thrombocytopoiesis and small numbers of functionally normal MKs (Alexander et al, 1996). Their haematopoietic progenitors are markedly reduced both in cell number and in their capacity to repopulate haematopoiesis in marrow-deficient mice (Solberg, 2005). In humans, elimination of a functional c-Mpl gene (located on chromosome 1p34) or the congenital absence of c-Mpl in children, results in severe thrombocytopenia due to decreased haematopoietic stem and lineage committed progenitor cells (Kaushansky, 2005a). Multiple mutations, including non-sense, frame-shift, splice-site alteration, missense or amino-acid substitutions, have been identified. These mutations result in a total absence of the c-Mpl receptor or functionally defective TPO-binding and/or signalling (van den Oudenrijn et al, 2002) with the type of mutation predicting the course of disease (Fig 3A) (King et al, 2005; Germeshausen et al, 2006). Congenital amegakaryocytic thrombocytopenia.

Fig 3. (A) Inherited thrombocytopenia due to disruptions of signalling molecules or transcription factors. Thrombocytopenia of different severities can result from varied inherited genetic mutations. This schema demonstrates the human mutations that are known to occur in the thrombopoietin (TPO) receptor c-Mpl or in transcription factor genes in megakaryocyte progenitor cells. The translated aberrant proteins have different disrupted functions which result in diseases characterised by thrombocytopenia and reduced platelet function. (B) Enhanced megakaryocyte proliferation and thrombocytosis due to aberrations of signalling molecules or transcription factors. Familial thrombocytosis can result from different inherited genetic mutations. This schema demonstrates the human mutations that are known to occur in TPO, its receptor c-Mpl, in the signalling molecule JAK2 or in the transcription factor GATA-1, in megakaryocyte progenitor cells. The translated aberrant proteins have different disrupted functions that result in diseases characterised by enhanced megakaryocyte progenitor proliferation and platelet production.
type I BM failure syndrome (CAMT I) in children is characterised by persistently low platelet counts (<20 x 10^9/l) and fast progression into pancytopenia (van den Oudenrijn et al, 2002). Haematopoietic stem-cell transplantation (HSCT) is currently the only curative treatment approach (Steele et al, 2005).

In CAMT type II, patients have delayed development of pancytopenia due to a residual activity of the TPO receptor (Ballmaier et al, 2003; Gandhi et al, 2005a; Gernshehausen et al, 2006). In contrast to the c-Mpl null and TPO null mice that maintain haematopoiesis and survive despite the 90% reduction in HSC and circulating platelets, humans with c-Mpl mutations have a lethal disease, which may be due to inherent differences in stem cell regulation and kinetics within the BM vascular niches.

An activating mutation within the transmembrane domain of the c-Mpl has been reported to be associated with familial essential thrombocythaemia (Ding et al, 2004). Interestingly, a c-Mpl polymorphism (Mpl Baltimore), with a single nucleotide substitution unique to individuals of African-American descent (7% heterozygous), is associated with reduced protein expression of Mpl, but a clinical phenotype of thrombocytosis (Moliterno et al, 2004). A similar decrease in c-Mpl receptor on the cell surface is seen in patients with polycythaemia vera (PV) and other myeloproliferative diseases (MPD). Thrombocytosis may therefore be related to the hypersensitivity to cytokines and signalling abnormalities found in these disorders (Kaushansky, 2005a).

Genetic mutations in the JAK2 gene are prevalent in patients with MPD (Tefferi & Gilliland, 2005). The molecular phenotype involving an activating JAK2 tyrosine kinase mutation, JAK2V617F, is a somatic point mutation causing the substitution of valine by phenylalanine in the protein product (James et al, 2005). Since a cytokine receptor scaffold is necessary for the transforming and signalling activities of JAK2, mutations can drive proliferative disease only in cells that express the type I cytokine receptors. In PV, the mutational frequency can be as high as 97% and in ET up to 57%, also being expressed in other myeloproliferative disorders (Baxter et al, 2005). At diagnosis, ET patients with the mutation have higher haemoglobin levels, increased white blood cell (WBC) count and an older age, harbouring a survival disadvantage and increased mortality (Wolanskyj et al, 2005). This recent discovery of the molecular basis of ET and other myeloproliferative disorders introduced great optimism for new therapeutic approaches in the near future.

Additional growth factors

While TPO is the main physiological regulator of megakaryocytopoiesis, it is not exclusive in this activity and is thought to function in conjunction with other factors (Kaushansky & Drachman, 2002). Other pleiotropic haematopoietic growth factors that stimulate MK growth alone or in combination with TPO include granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-6, IL-11, stem cell factor, FLT ligand, fibroblast growth factor (FGF) and EPO (Tomer et al, 1987, 1988; Broudy & Kaushansky, 1995; Deutsch et al, 1995; Kaushansky et al, 1995; Bruno et al, 2003).

A novel MK growth stimulating peptide has recently been described, which drives robust proliferation of CD34+ haematopoietic progenitor cells and MK in vitro and MK progenitors in transgenic mice via protein kinase C (PKC) signalling pathways. This unique peptide is derived from the cleavable C-terminus of the stress associated form of acetylcholinesterase (AChE), a molecule known to be involved in the regulation of megakaryocytopoiesis (Long et al, 1982; Lev-Lehman et al, 1997). This readthrough variant (AChE-R), is physiologically functional during stress thrombopoiesis and is currently in preclinical development as a new thrombopoietic factor (Grisaru et al, 2001; Deutsch et al, 2002; Pick et al, 2005).

Negative regulators of megakaryocytopoiesis

Several factors are known to inhibit MK development, including transforming growth factor-β1 (Kuter et al, 1992), PF4 and IL-4 (Han et al, 1991; Zauli & Catani, 1995). Src kinase inhibitors have recently been shown to negatively regulate MK proliferation by inducing MK differentiation and functional platelet-like fragment formation in vitro (Gandhi et al, 2005b; Lennuitti et al, 2005). This important discovery may enable new therapeutic modalities for treating thrombocytosis.

Cellular interactions and chemokines

Megakaryocytopoiesis and thrombocytogenesis require cytokines, chemokines and cellular interactions between HSC, and marrow stromal cells (Avecilla et al, 2004). Stromal-derived factor-1 (SDF-1), of the CXC family, is the key chemokine involved in the retention of haematopoietic precursor cells in the BM. SDF-1 supports megakaryocytopoiesis and homing of HSCs to the BM during foetal development (Wang et al, 1998). SDF-1 is produced locally by the stromal cells, promotes the migration and contact of immature MK with a permissive, endothelial-enriched BM environment (Broxmeyer et al, 2004) and functions during stress thrombopoiesis and is currently in preclinical development as a new thrombopoietic factor (Grisaru et al, 2001; Deutsch et al, 2002; Pick et al, 2005).
MAPK and AKT MAPK pathways, which include extracellular signal-related kinase (MEK) and extracellular signal-regulated protein kinase (ERK)1/ERK2, to regulate cell proliferation and differentiation. The molecular upregulation of the CXC cytokine, CTAP III, in MKs is associated with maturation and, as with other chemokines, is involved in cellular interactions with extracellular matrix and platelet production (Deutsch et al., 2000).

**Nuclear transcription factors**

Megakaryocyte development and thrombocytopenia are controlled by the concerted action of TFs, which form complexes that co-ordinately regulate the chromatin organisation to specifically activate the genes of MK lineage precursors and/or concurrently repress gene expression that support other cell types. Many MK-specific genes are co-regulated by GATA and Friend of GATA (FOG) together with acute myeloid leukaemia/runt-related TF 1 (AML/ RUNX1) and ETS proteins. Mutations in TF genes result in the translation of aberrant proteins that cause thrombocytopenia or thrombocytosis of various degrees. The inherited changes and disorders that result are summarised in Fig 3A and B. RUNX1 (or AML1) is essential for generation of all definitive haematopoietic lineages. The RUNX1/AML-1 core-binding factor (CBFβ) complex binds the N-terminal transcription activation domain of GATA-1, which enables the programming of MK lineage commitment (Elagib et al., 2003). Chromosomal translocations in the RUNX1 gene is frequently associated with leukaemia, for example the t(8;21) in AML resulting in a dominant-negative RUNX1-ETO fusion protein. Germ line mutations of RUNX1, which cause monoallelic loss of RUNX1 complexes, are found in the autosomal dominant familial platelet disorder (FDP) with MK maturation arrest and the prominent appearance of disorganised internal membranes, reduced granule numbers, and severe platelet deficit and display defective inside-out signalling by the αIIbβ3 integrin (Shiraga et al., 1999).

Megalakaryocytic and erythroid lineages, which are derived from a common bipotential progenitor, share many early lineage-restricted TFs that support other cell types. Many MK-specific genes are co-regulated by GATA and Friend of GATA (FOG) together with acute myeloid leukaemia/runt-related TF 1 (AML/ RUNX1) and ETS proteins. Mutations in TF genes result in the translation of aberrant proteins that cause thrombocytopenia or thrombocytosis of various degrees. The inherited changes and disorders that result are summarised in Fig 3A and B. RUNX1 (or AML1) is essential for generation of all definitive haematopoietic lineages. The RUNX1/AML-1 core-binding factor (CBFβ) complex binds the N-terminal transcription activation domain of GATA-1, which enables the programming of MK lineage commitment (Elagib et al., 2003). Chromosomal translocations in the RUNX1 gene is frequently associated with leukaemia, for example the t(8;21) in AML resulting in a dominant-negative RUNX1-ETO fusion protein. Germ line mutations of RUNX1, which cause monoallelic loss of RUNX1 complexes, are found in the autosomal dominant familial platelet disorder (FDP) with MK maturation arrest and the prominent appearance of disorganised internal membranes, reduced granule numbers, and severe platelet deficit and display defective inside-out signalling by the αIIbβ3 integrin (Shiraga et al., 1999).

The proto-oncogene c-myc, which functions together with p300 as an early mandatory haematopoietic TF, is also a powerful negative regulator of thrombopoiesis in mice (Metcalf et al., 2005). Impairment of its association with the transcriptional coactivator p300 increased numbers of HSCs, MKs and platelets (Sandberg et al., 2005). Mpt1/− mice harbouring mutations in the DNA-binding domain (called p303) or within the leucine zipper domain (called p43) of the c-Myc gene, exhibit TPO independent excessive thrombopoiesis (Carpinelli et al., 2004).

**Platelet production and release**

During MK maturation, internal membrane systems, granules and organelles are assembled in bulk. Platelet production by cytoplasmic fragmentation requires highly structured intricate changes in the MK cytoskeleton and concomitant assembly of anucleate platelets. High ploidy MKs form extensive internal demarcation membrane, which is continuous with the plasma...
membrane and serves primarily as a membrane reservoir for the formation of the precursor of cytoplasmic extensions called proplatelets. The open canalicular system, a channelled system for granule release and a dense tubular network, is formed prior to the initiation of proplatelet assembly (Patel et al., 2005). Some proplatelet proteins, such as VWF and fibrinogen receptors, are synthesised and directed to the MK surface membrane while others proteins are conveyed into secretory granules. Individual organelles migrate from the cell body to the proplatelet ends, with approximately 30% of organelles/granules in motion at any given time (Richardson et al., 2005). Fibrinogen is taken up by MK from the plasma through endocytosis and/or pinocytosis and selectively transferred to platelet-specific granules.

Quantitative electron microscopic analysis has shown that MKs are located <1 micron away from the marrow sinus wall allowing access of the newly formed platelets to the circulation (Lichtman et al., 1978). Because an entire MK may pass through transendothelial apertures of 6 microns diameter, some MKs reach the lungs through the circulation (Tavassoli & Aoki, 1981), which may provide an alternative site for platelet production (Eldor et al., 1989; Zucker-Franklin & Philipp, 2000). Nascent platelets contain mitochondria and ribosomal RNA (reticulated platelets), and all the components necessary for platelet function in haemostasis, but do not contain nuclear material (Patel et al., 2005).

Currently, there are two models of thrombopoiesis, which are not mutually exclusive. One model proposes platelet assembly and budding off the tips of proplatelets, which operate like assembly lines for platelet production at the end of each proplatelet (Patel et al., 2005). The proplatelets extend into sinusoidal spaces, where they detach and fragment into individual platelets, giving rise to about 2000–5000 new platelets (Zucker-Franklin, 1970; Choi et al., 1995). The other model of platelet biogenesis proposes that, within the MK cytoplasm, there are preformed territories with internal membranes demarcating prepackaged platelets that are released upon fragmentation of the cytoplasm. This theory is based on electron microscopy analysis of the internal membranes of MKs (Zucker-Franklin & Petursson, 1984; Mori et al., 1993). Mature MK, when cultured in contact with subendothelial extracellular matrix, are stimulated to produce platelets by a highly efficient explosive fragmentation of the entire cytoplasm (Caine et al., 1986; Eldor et al., 1986). In vivo and ex vivo observations of platelet release from MKs with phase-contrast microscopy strongly support the explosive-fragmentation theory (Deutsch et al., 1995; Kosaki, 2005).

**Disorders of thrombopoiesis**

Disorders of platelets are diverse and can be genetic or acquired. Inherited disorders usually result from genetic mutations within genes that encode regulatory proteins, as mentioned above, or proteins necessary for platelet production, and can have severe clinical results with profound thrombocytopenia and extended bleeding times. May–Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, Alport syndrome, and Epstein syndrome are related to mutations in the MYH9 non-muscle myosin heavy chain IIA gene, which encodes the sole myosin isoform expressed in platelets and neutrophils (Balduini et al., 2002). The defect in myosin causes altered cytoskeletal dynamics resulting in macrothrombocytopenia. Bernard–Soulier syndrome is an autosomal dominant disorder characterised by a prolonged bleeding time, macrothrombocytopenia, defective prothrombin consumption and poor platelet adhesion. The dysfunction is due to the absent or deficient expression of the GPIb/IX/V complex, which forms the VWF receptor on the platelet surface (Caen & Rosa, 1995).

Several mutations within the genes that encode GP Ibα, GPV, or GPIX have been identified (Patel et al., 2005). Gray platelet syndrome is another autosomal dominant disease characterised by macrothrombocytopenia with absence of α-granules. This abnormality is due to inadequate packaging of VWF and fibrinogen within the α-granules, which fail to mature during MK differentiation. The clinical and molecular heterogeneity in this syndrome has been recently reviewed (Nurden & Nurden, 2006).

**Clinical use of thrombopoietic agents**

It was expected that the physiologically powerful stimulators of thrombopoiesis, rHuTPO, or the truncated cloned rHuMGDF, would be effective in patients with thrombocytopenia responsive to exogenous stimulation of platelet production. Although in non-human primates the administration of rHuTPO or PEG-rHuMGDF resulted in marked responses (Tomer & Harker, 1996; Harker et al., 1997) (Fig 2), in human trials performed with the truncated PEG-rHuMGDF, cross-reacting neutralising antibody to endogenous TPO developed, which led to cessation of the trials (Basser, 2002; Solberg, 2005). In addition, although rHu-TPO facilitated the collection of platelets in normal donors and patients for transfusion purposes (Kuter et al., 2001) in thrombocytopenic patients, there was no positive effect on platelet recovery following aggressive chemotherapy (Kaushansky, 1995). The administration of rHuTPO before or after stem cell transplantation and myeloablative therapy had no impact on the duration of severe thrombocytopenia, nor did it reduce the requirement for platelet transfusions either post-transplant or in AML patients (Bernstein et al., 2002; Nash et al., 2002; Schuster et al., 2002). The failure of escalated TPO levels to stimulate platelet production may be related to the paucity of MK progenitor population (Scheding et al., 2002; Vadhan-Raj et al., 2005). In a recent phase I study by the Children’s Oncology Group, Children’s National Medical Center, children with solid tumours received rHuTPO with granulocyte colony-stimulating factor (G-CSF) after combination chemotherapy. The rHuTPO was well tolerated and various doses resulted in a median time to platelet recovery (>100 × 10^9/l) of 22–25 d. Time to haematological recovery and median number of platelet transfusions was somewhat improved compared with historical controls receiving combi-
nation chemotherapy with G-CSF (Angiolillo et al, 2005). A recent review of the use of thrombopoietic factors in chronic BM failure states, suggests that although the temporal pace of response appears to be relatively slow, durable responses can be achieved in patients given thrombopoietic factors for extended periods of time (Kurzrock, 2005). Thus, TPO may prove useful in thrombocytopenic states where stem cells have not been severely depleted and the marrow microenvironment can support thrombopoietic activity.

Human trials have been performed with IL-6 and IL-11. IL-6 is a pleiotropic cytokine with potent thrombopoietic activity (Stahl & Zucker-Franklin, 1993) and its administration to humans markedly increased platelet counts (Hoffman, 1989) and accelerated platelet recovery post autologous peripheral blood stem cell transplantation (Nagler et al, 1995). Unfortunately, IL-6 administration involved severe toxicities (Kurzrock, 2005). The multilineage cytokine IL-11 has been shown to attenuate chemotherapy-induced thrombocytopenia (Kurzrock, 2005) and the administration of low-dose IL-11 raised platelet counts without significant toxicity in selected patients with BM failure (Kurzrock et al, 2001). Currently, IL-11 (Neumega) is the only clinically approved drug for treating platelet counts without significant toxicity in selected patients without significant toxicity in selected patients with BM failure (Kurzrock et al, 2001). Currently, IL-11 (Neumega) is the only clinically approved drug for treating thrombocytopenia (Orazi et al, 1996), but has limited effects with no clear cost benefit over platelet transfusions (Cantor et al, 2003; Kurzrock, 2005).

Currently, exciting studies with the new thrombopoietic agents have invigorated the field, with positive results reported in idiopathic thrombocytopenic purpura and other disorders (Solberg, 2005). AMG531 is an engineered peptide with no sequence homology to TPO. It is composed of a recombinant protein carrier Fc domain linked to multiple Mpl-binding domains and has similar effects on megakaryocytopoiesis to that of TPO. Kuter (2005) described a phase II clinical trial of 21 immune thrombocytopenic purpura (ITP) patients treated with AMG531 for 6 weeks with platelet counts that increased in most patients to greater than 50 × 10^9/L. The recent safety profile of AMG531 summarises two phase 1 studies in a total of 56 healthy volunteers and three phase 2 studies, with the option to continue in an open-label extension study, in a total of 57 patients with ITP. No clinically important events or detectable neutralising antibodies were reported. However, across the phase II studies, three patients experienced worsening of thrombocytopenia, one patient had elevated lactate dehydrogenase and one patient, diffuse reticulin formation in the BM (Stepan et al, 2005).

Another phase I trial with a novel, pegylated TPO-mimetic peptide (peg-TPOmp), was described by Cerneus et al (2005) in which 40 volunteers were randomised to receive this TPO receptor agonist or placebo as a single i.v. bolus. Platelet counts increased dose-dependently, reaching peak levels at day 10–12, and returned to baseline within 3–4 weeks, with mean peak platelet levels ranging from 315 × 10^9 to 685 × 10^9/L, and increments of 1.4-fold to 2.2-fold. Platelet function was not different between the treatments. No serious adverse events and no antibodies against peg-TPOmp were detected.

A minibody agonist of the c-Mpl was recently used in a novel therapeutic approach to treat thrombocytopenia (Orita et al, 2005). IgG antibodies were engineered against the TPO receptor with absent or very weak agonist activity, to be agonist minibodies, which include diabody or single chain (Fv2) [sc(Fv)2] as strong as the natural ligand. Two minibodies were successfully constructed to bind and activate two types of dysfunctional mutant of Mpl receptor that cause CAMT. This conversion of biological activities by designing minibodies can be widely applicable in the future to generate agonist minibodies for other clinical applications.

Three new synthetic c-Mpl agonists under preclinical development have recently been described. One is an orally active, nonpeptidyl TPO receptor agonist, YM477 (Suzuki et al, 2005). Its effect was examined in human platelet-producing non-obese diabetic severe combined immunodeficient mice in which human HSCs were transplanted. Oral administration of YM477 dose-dependently increased the number of human platelets produced by these mice, reaching an increase of about 3.0-fold on day 14. The other synthetic c-Mpl agonist, NIP-004, induced the proliferation of various TPO-responsive cell lines, primary human hematopoietic progenitor cells and human thrombocytopoiesis in a xenotransplant animal model, showing 1.5-fold increase of HuCD45⁻CD34⁺CD41a⁺ megakaryoblasts and threefold increase of HuCD41a⁺ 128 N matured polyploid MKs in murine BM. NIP-004 increased the circulating HuCD41a⁺ platelets by sixfold (Nakamura et al, 2006). The efficacy and safety of these molecules in clinical settings has yet to be determined. SB-497115 is a non-peptidyl TpoR agonist that is being developed by GlaxoSmithKline to treat thrombocytopenia. Data from a phase I study in healthy volunteers, show that SB-497115 increased platelet counts in a dose-dependent fashion when administered orally (Jenkins et al, 2004). Patients are currently being recruited in phase II clinical trials.

In conclusion, the recently acquired knowledge of the molecular mechanisms that regulate megakaryocytopoiesis and platelet production, together with the new developments in the generation and availability of various thrombopoietic agents, provide a strong basis for optimism in the future therapy of thrombocytopenic states.

Acknowledgements

VRD acknowledges research funding from the Israel Ministry of Science and the BMBF, Germany, The Israel Cancer Association – donation of Don and Donna Sherman and The Israel Ministry of Health. AT acknowledges support from Lyonel G Israels fund, Winnipeg, Manitoba, Canada.

References

Review


