Thrombopotein: its ups and downs

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Chapter 1

General Introduction
## CONTENTS

1.0 Introduction ......................................................................................................................... 11

1.1 Platelets ............................................................................................................................. 11
   1.1.1 Platelet function .......................................................................................................... 11
   1.1.2 Platelet structure ........................................................................................................ 12
   1.1.3 Platelet abnormalities ............................................................................................... 13

1.2 Megakaryocytopoiesis ........................................................................................................ 14

1.3 Thrombopoietin .................................................................................................................. 15
   1.3.1 Discovery .................................................................................................................... 15
   1.3.2 Structural Characteristics .......................................................................................... 16
   1.3.3 Tpo production sites ................................................................................................. 17
   1.3.4 Regulation of Tpo production ..................................................................................... 18
   1.3.5 Biological properties ................................................................................................. 18
     1.3.5.1 In vitro and ex vivo effects ..................................................................................... 18
     1.3.5.2 In vivo effects ........................................................................................................ 19

1.4 The Tpo receptor, Mpl ....................................................................................................... 23
   1.4.1 Discovery and structural characteristics .................................................................... 23
   1.4.2 Expression of the Tpo receptor, Mpl ........................................................................ 24
   1.4.3 Signal transduction ..................................................................................................... 25

1.5 Controlling thrombocytopoiesis by regulation of thrombopoietin levels ......................... 26
   1.5.1 Fixed Tpo production and c-Mpl-mediated Tpo uptake ............................................ 26
     1.5.1.1 Platelets bind and metabolise Tpo .................................................................... 27
     1.5.1.2 Megakaryocytes participate in Tpo clearance .................................................... 27
     1.5.1.3 Tpo production is constitutive: evidence against transcriptional regulation ... 28
   1.5.2 Evidence in favour of transcriptional regulation and other forms of Tpo regulation 28

1.6 Measurement of Tpo levels as a diagnostic tool ............................................................... 29

1.7 Outline of this thesis .......................................................................................................... 29

1.8 References ......................................................................................................................... 30
1.0 Introduction

Already in 1958 Kelemen et al.[1], postulated that a factor existed that is responsible for the regulation of the production of blood platelets, or thrombocytes. In analogy with the blood hormone erythropoietin (Epo), which is responsible for erythrocyte production, the name thrombopoietin (Tpo) was proposed. It took until 1994 for this regulator to be cloned. With the discovery of thrombopoietin, the field of mega- and thrombocytopoiesis, the regulation of platelet production, entered a new era. Many studies were performed to gain insight into the biochemical features of thrombopoietin. Its regulation and physiological functions were addressed and the diagnostic value of thrombopoietin measurements in blood was investigated. Within a year after the discovery of thrombopoietin, (pre)clinical studies with recombinant preparations were initiated to evaluate its therapeutic efficacy. Before the discovery of thrombopoietin many factors with thrombopoietic capacity were tested for the treatment of thrombocytopenia but none sufficed. In contrast to these factors, thrombopoietin is lineage specific and has the capacity to support both proliferation and maturation of cells from the megakaryocytic lineage. So far, administration of recombinant thrombopoietin to patients seems safe and well tolerated. However, in some individuals neutralising antibodies against Tpo developed upon repeated administration of PEG-rHuMGDF, a modified form of thrombopoietin. All clinical trials with this preparation have therefore been aborted.

Tpo is effective in increasing the platelet counts in some, but not in all, thrombocytopenic states. Especially the benefit of thrombopoietin administration in conjunction with myelosuppressive therapy was limited. Although platelet counts in these patients increased upon therapy, the duration and severity of the thrombocytopenia were not significantly improved. In case of myelosuppressive therapy, and possibly also in other patient groups, ex vivo expansion of megakaryocytic progenitors seems to hold a clinical promise. Apart from its therapeutic application, analysis of Tpo levels in various disorders represents a valuable diagnostic tool. In addition, it gives insight into the regulation of thrombocytopoiesis and the interplay between platelets and Tpo. In this thesis, these topics are addressed in detail. This chapter gives an overview on the current knowledge of thrombopoietin and its receptor (Mpl), and its role in thrombocytopoiesis, which forms the basis of the research described in this thesis.

1.1 Platelets

1.1.1 Platelet function

Platelets, or thrombocytes, are small nonnucleated blood components that are involved in many physiological and pathological processes [2]. They play an essential role in haemostasis, i.e. the control of haemorrhage and restoration of the vascular system in case of injury. When the vascular endothelium is damaged, platelets adhere to the exposed subendothelium, undergo a shape change, and secrete their granule contents (release reaction). With the release of ADP and thromboxane \( A_2 \), other passing platelets are "recruited" at the site of tissue damage and platelet-to-platelet interactions eventually result in the formation of an aggregate, the
so-called haemostatic plug. The released factors display a broad range of activities. Functions of secreted factors include the promotion of vasoconstriction (thromboxane A₂, serotonin) and vascular healing (platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)). In addition, phospholipids (platelet factor 3) expressed on the platelet cell surface after activation, and secreted factors such as factor V (FV), von Willebrand Factor (vWF), platelet factor 4 (PF4) and fibrinogen, are involved in the activation and/or promotion of the coagulation cascade at the site of injury.

1.1.2 Platelet structure
Platelets form the smallest cellular blood elements. Reported sizes vary from less than 4 μm to over 12 μm [3,4]. A schematic representation of a platelet is shown in Figure 1. Platelets normally circulate as flattened discs. Their shape is maintained by a circumferential microtubuli structure and submembranous filaments. Platelets have a sponge-like appearance. The plasma membrane invaginates to the interior of the cell, forming a surface-connected open canalicular system (OCS), thus highly increasing the surface area of the platelet. On the phospholipid membrane, several glycoprotein (GP) receptors are expressed. These receptors play an important role in haemostatic mechanisms, such as the aforementioned adhesion, aggregation and coagulation. Three major glycoprotein complexes can be distinguished. The glycoproteins IIb and IIIa form a complex (GPIIb/IIIa; about 80,000 copies per platelet) that, upon activation, functions as a binding site for fibrinogen. When adjacent platelets bind fibrinogen, bridges are formed, which results in platelet aggregation. In addition, the GPIIb/IIIa has activation-dependent binding sites for adhesive proteins such as fibronectin and vWF [5]. The glycoprotein complex GP Ib/IIa (900-2300 copies per platelet) functions as a receptor for the matrix protein collagen [6]. The third complex, GPIb/IX/V (about 25,000 copies per platelet) is mainly involved in adhesion. Under high shear stress conditions, GPIb/IX/V binds to the exposed subendothelium of damaged vessel walls by binding to surface-bound vWF [7]. The GPIb molecule of this complex consists of two disulphide-linked chains, the α-chain and the
β-chain (Fig. 2). The vWF binding domain is located on the α-chain of GPIb, as is a binding site for thrombin [8]. The extramembranous portion of the alpha chain, which is designated glycocalicin (GC) can be proteolytically cleaved from the intact molecule [9]. It has been shown that GC circulates in the blood and can be used as a marker for overall platelet turnover [10-12].

Also expressed on the cell surface, is the receptor for Tpo, Mpl [13-15]. With this receptor, platelets can bind and internalise Tpo [14-17]. The significance of this process will be discussed in more detail in § 1.5 and in the following chapters.

Platelets contain two types of storage granules, α-granules and electron dense granules (δ-granules). The contents of these granules are secreted when platelets become (sufficiently) activated. Upon activation, platelets undergo a series of changes, which include a shape change from a discoid to a spherical appearance, movement of the granules to the centre of the platelet within a ring of microtubuli, the formation of pseudopods and the release of the granule content in the OCS. The α-granules contain procoagulants (e.g. fibrinogen, vWF), antiheparins (e.g. platelet factor 4, β-thromboglobulin) and growth factors (e.g. PDGF, thrombospondin). In the δ-granules, serotonin and nucleotides (ADP, ATP) and cations (Ca^{2+} and Mg^{2+}) are contained. In addition to storage granules, platelets also contain lysosomes and peroxisomes. Glycogen granules and mitochondria are responsible for the energy metabolism and submembranous filaments represent the platelet's contractile system.

1.1.3 Platelet abnormalities

In healthy individuals, circulating platelet numbers range from 150-450x10^9 per litre of blood. The life-span of a platelet is approximately 10 days. In order to maintain normal numbers of
circulating platelets, it is essential that there is a balance between platelet production and platelet removal. Disruption of this balance may lead to serious haemostatic disorders. A lack of platelets, i.e. thrombocytopenia, may result from a decreased platelet production, platelet sequestration, a shortened platelet life-span or an increased platelet consumption. Thrombocytopenia may cause an abnormal bleeding tendency resulting in haemorrhage. Similar problems may occur when platelet function is impaired, such as occurs in Glanzmann's disease (hereditary platelet GPIIb/IIIa deficiency) or Bernard-Soulier Syndrome (hereditary platelet GPIb/IX/V deficiency). Abnormally high levels of circulating platelets, thrombocytosis, may lead to thrombosis and/or haemorrhage. Elevated platelet levels occur for instance in patients with hereditary thrombocythaemia or in patients with myeloproliferative disorders such as polycythaemia vera or essential thrombocythaemia.

1.2 Megakaryocytopoiesis

Platelets originate from megakaryocytes. Megakaryocytes are large multinucleated, polyploid cells that reside in a low frequency in the bone marrow. Megakaryocytes are formed after a complicated process of proliferation, differentiation and maturation of pluripotent haematopoietic stem cells during a process called megakaryocytopoiesis (for review see [18-21]). The pluripotent haematopoietic stem cells have the capacity of self-renewal and form the basis for the production of blood cells from all different lineages. Production of the different cell lineages is controlled by both broadly acting haematopoietic growth factors (often called colony-stimulating factors) and cell-line specific growth factors. Figure 3 shows a schematic representation of megakaryocytopoiesis and the formation of platelets. The pluripotent stem cell proliferates and progresses via a common precursor for the erythroid and the megakaryocytic

Figure 3: Schematic overview of megakaryocytopoiesis. Tpo exerts its function on stem cells and megakaryocytic progenitor cells. Upon binding to the Tpo-receptor, Tpo induces proliferation, differentiation and maturation of these cells which leads to the formation of proplatelets. Finally, proplatelets disintegrate into platelets. During the maturation process polyploidisation takes place which is indicated with the numbers above the cells.
lineage to the colony-forming unit megakaryocyte (CFU-Meg). This CFU-Meg proliferates and differentiates into megakaryoblasts which, in turn, develops into the megakaryocyte. Megakaryocytes undergo a series of endomitotic replications, which involves DNA replication and the increase in cytoplasmic volume without cell division. In this way, very large multinucleated cells are formed, of which the chromosome number can be as high as 64N or 128N. This process is unique for megakaryocytes. During maturation, demarcation membranes form in the cytoplasm, which ultimately lead to the formation of proplatelets. Proplatelets are long cytoplasmic segments that fragment into platelets. One megakaryocyte can produce as many as 4000 to 6000 platelets. Per day, approximately $10^{11}$ platelets are formed to maintain a normal platelet count [150-450x10$^9$/L] in the circulation.

1.3 Thrombopoietin

1.3.1 Discovery

Although the existence of a factor with thrombopoietic capacity was suggested some decades ago [1,22], its cloning and isolation had to be awaited for until 1994. Since the sixties, it was known that plasma, serum and urine of thrombocytopenic animals and humans possessed thrombopoietic activity [1,22-31]. The name "thrombopoietin" was postulated for the factor responsible for this activity. Many attempts were made to biochemically isolate thrombopoietin from these body fluids [27-30,32] or from conditioned medium from cultured cell lines [33,34]. In the mean time, the thrombopoietic capacity of several other cytokines such as interleukin-1 (IL-1), IL-3, IL-6, IL-11 and leukemia-inhibiting factor (LIF) was reported, but none of these factors had the potential to stimulate the entire megakaryocyte development [35].

The eventual isolation of thrombopoietin came from an unexpected direction. In 1986, Wendling and co-workers reported the discovery of a murine retrovirus, the myeloproliferative leukemia virus (MPLV), that induced a myeloproliferative syndrome in mice [36]. Some years later, the oncogene responsible for this transformation, v-mpl [37], was identified which was followed by the cloning of the cellular homologue c-mpl [38]. Sequence data revealed the presence of two haematopoietic growth factor receptor motifs, which strongly suggested that c-mpl encoded a new haematopoietic growth factor receptor. The importance of this receptor and its ligand in megakaryocytopoiesis was soon recognised as RT-PCR analysis showed that the receptor was expressed exclusively on cells from the megakaryocytic lineage. Moreover, c-mpl antisense oligonucleotides were shown to inhibit megakaryocyte colony formation in vitro. This inhibition was specific, since colony formation for other cell lineages remained unaffected [39].

The cloning of thrombopoietin by five separate groups [40-44] put an end to the long search for the stimulator of platelet production. Thrombopoietin (Tpo), also designated Mpl Ligand (ML), Megakaryocyte Growth and Development Factor (MGDF) and megapoietin, proved to be the most important factor in the regulation of platelet production.
1.3.2 Structural characteristics

The gene for Tpo is highly conserved between different species [40,44,45]. Human Tpo maps to the long arm of chromosome 3, at 3q27-28 spanning approximately 6 kb [45-49]. The coding region consists of 6 exons and 5 introns and encodes a protein of 353 amino acids (figure 4). The first 21 amino acids form a signal peptide that is cleaved off to generate the mature polypeptide. Within the mature Tpo protein two domains can be distinguished: the amino-terminal and the carboxy-terminal domain. The N-terminal domain comprises 153 amino acids, and bears 23% sequence homology with erythropoietin (Epo) and 50% similarity when taking conservative substitutions into account [40,44,45]. Similar to Epo, it contains four cysteine residues that can form two disulphide bridges. This domain bears more than 80% homology with porcine, murine and rat Tpo and has two receptor-binding sites. Functional truncation studies showed that this amino-terminal domain is responsible for the hormonal activity of Tpo [40,44]. The carboxy-terminal region consists of 179 amino acids and is more unique. It has no sequence similarity with other known proteins and is less conserved between the species. It contains six potential N-linked and several O-linked glycosylation sites [40,44]. Glycosylation of these sites might explain the discrepancy between the predicted molecular weight of 38 kD based on the protein sequence and the reported molecular weights of around 80 kD [50]. Masses smaller that 38 kD have also been reported, suggesting that post-translational processing of the Tpo protein might also occur [35]. The C-terminal domain, in particular the N-glycosylation sites, was shown to play a role in the secretion of the Tpo protein [51,52]. In addition, in analogy with the glycosylated form of Epo [53,54], it has been suggested that the C-terminal domain plays a role in stabilisation and enhancement of the circulating half-life of Tpo.

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**Figure 4:** Genomic organization of the coding region of the Tpo gene (A) and the Tpo protein structure (B). Panel A depicts the 6 exons. Panel B represents the protein structure of Tpo. Cys refers to the presence of cysteine residues, whereas the asterisks represent the 6 N-glycosylation sites in the C-terminal domain. bp: base pairs; aa: amino acids.
Apart from the full-length form of Tpo, in humans, two different isoforms, Tpo-2 and Tpo-3, have been identified. Both result from alternative mRNA splicing in exon 6, deleting 12 bp and 116 bp respectively [47,55,56]. They are present in similar frequencies in the Tpo producing organs and are conserved in pigs and mice [16,47,55]. The exact function of these isoforms is unknown. Tpo-2 was found to be poorly secreted and did not induce proliferation of Ba/F3-mpl cells [47]. Possibly, these isoforms have a regulatory function. Analysis of different human carcinoma cell lines revealed the existence of another three isoforms, designated Tpo-4, Tpo-5 and Tpo-6. Transfection of Tpo-5 and Tpo-6 cDNA in COS-1 cells did not result in the secretion of biologically active protein. Tpo-6 was not produced at all and Tpo-5 was poorly secreted. The activity of Tpo-4, of which the equivalent is present in mice, was not assessed [56].

1.3.3 Tpo production sites

The main production sites of Tpo in both humans and animals, as determined by the number of Tpo transcripts, are the liver and, to a lesser extent, the kidney. In addition, Tpo mRNA transcripts have been detected in various other organs and tissues, such as the bone marrow, the spleen, the brain and the placenta [16,40,41,44,45,47,55,57-65]. In situ hybridisation studies have shown that in the liver, parenchymal and sinusoidal endothelial cells are responsible for Tpo production [61,66,67]. In addition, several hepatoblastoma cell lines have been shown to produce biologically active Tpo [68,69]. In the kidney, Tpo production is confined to the cells of the proximal convoluted tubuli [61]. Production of biologically active Tpo by a human embryonic kidney cell line has been reported [70-72].

It is generally accepted that the liver is the primary source of Tpo production. Recently, it was reported that normal mice, transplanted with the liver of Tpo knockout mice, had a 50 to 60 percent reduction in their circulating platelet numbers [73]. Thus, at least in mice, it seems that a large part of the Tpo production is accounted for by the liver. Also in humans, several in vivo studies support the importance of the liver as a Tpo-producing organ. Patients with severe liver disease often suffer from thrombocytopenia, which can be resolved by orthotopic liver transplantation. Follow-up of Tpo levels in thrombocytopenic patients with severe liver cirrhosis, causing impaired liver function, has shown that Tpo levels are not elevated, despite the need for increased thrombocytopoiesis. Upon orthotopic liver transplantation, Tpo levels rose and returned to normal when platelet counts were increasing [74-80]. This suggested that upon engraftment of the new liver, Tpo production was restored. However, apart from a decreased Tpo production, platelet pooling in the spleen might also contribute to the thrombocytopenia in these patients. Patients with liver cirrhosis often have an enlarged spleen.

In patients with a liver cell tumour or hepatoblastoma, high levels of circulating Tpo and thrombocytosis have been reported [81-83]. Compared to the contribution of the liver to Tpo production, the contribution of the kidney seems to be less significant. Patients with chronic kidney failure do not have a major decrease in platelet count or Tpo levels (see also Chapter 4) [84].
1.3.4 Regulation of Tpo production

In vivo studies indicate that Tpo production by the liver and kidney is constitutive. Upon induction of thrombocytopenia in animal models and in Tpo-receptor knock-out or so-called mpl−/− mice, transcription of the Tpo gene was not enhanced, nor was there a shift in the generation of alternative splice transcripts [16,47,61,65,85-88]. In contrast, bone-marrow stromal cells seem to produce Tpo in a non-constitutive fashion. Tpo mRNA levels were increased in bone-marrow stromal cells from patients with thrombocytopenia due to aplastic anaemia, chemotherapy or autoantibodies [61,63]. The exact factor(s) responsible for the enhancement of Tpo transcription in these cells is unknown, although it was demonstrated in two recent reports that platelet α-granular proteins such as PDGF, FGF, PF4, TSP and TGF-β could influence the Tpo production by BM stromal cells [64,89]. Sakamaki et al. [89] demonstrated with Taqman real-time RT-PCR analysis, that TGF-β could enhance Tpo mRNA transcription in bone-marrow stromal cell cultures. The concentration of Tpo in these cultures was also increased. Other factors such as IL-6, G-CSF, Epo, IL-3 or TNF-α were not effective. TGF-β is stored in and can be released by megakaryocytes and platelets [90,91]. It has a suppressive effect on the formation of megakaryocyte-colony forming units (CFU-Meg) [92,93]. Therefore, the authors hypothesised that a feedback mechanism might exist in which TGF-β released from destroyed platelets or megakaryocytes stimulates the synthesis of Tpo by bone-marrow stromal cells. The production of Tpo leads to enhanced megakaryocytopoiesis. With the progression of megakaryocytopoiesis, the expression of the TGF-β receptor on these cells will increase as well. This eventually will render them susceptible for suppression by TGF-β. The validity of this model remains to be investigated. In contrast to the Tpo-upregulation under the influence of TGF-β, reported by Sakamaki et al. [89], Sungaran et al. [64] reported a negative modulation of Tpo mRNA expression in BM stromal cells by this factor. PF4 and TSP were also found to suppress Tpo mRNA expression, whereas PDGF and FGF increased Tpo mRNA expression and the levels of Tpo protein in the supernatant. The conflicting results with respect to the effect of TGF-β reported by Sakamaki et al. [89] and Sungaran et al. [64] might (partly) be due to the incubation period of the cultures, 24 hrs versus 4 hrs, or to the differences in methods of quantification, quantitative PCR versus semi-quantitative PCR analysis.

Further studies should clarify which factors are involved in the regulation of Tpo production by bone-marrow stromal cells and in what fashion.

Although the local Tpo production by BM stromal cells might prove to be of importance in certain pathological states, notably in immune mediated thrombocytopenias, its contribution to the overall Tpo production is probably very low. When compared with the number of Tpo transcripts in liver and kidney, the number of Tpo transcripts in the bone marrow is low.

1.3.5 Biological properties

1.3.5.1 In vitro and ex vivo effects

Both in vitro and in vivo studies provide ample evidence that Tpo is indeed the main regulator of thrombocytopoiesis. In vitro studies in which the effect of recombinant Tpo on megakaryo-
cyte formation and development was assessed have shown that Tpo is involved in the proliferation, differentiation and maturation of megakaryocytic progenitor cells up to the induction of proplatelet formation (see Fig. 3) [40,43,44,59,94-97]. In serum-free cultures [94,95,98] and single-cell cultures [95,99] it was demonstrated that Tpo could establish the whole process of megakaryocytopenesis, independent of the presence of other cytokines or accessory cells. Although other cytokines lack this capacity, for optimal \textit{ex vivo} expansion of megakaryocytic progenitors, Tpo should be added in culture together with other cytokines [94,95,100-112]. Platelets formed in culture under the influence of Tpo were shown to be of normal morphology and completely functional [113,114]. The absence of Tpo, rather than the presence of Tpo, seems to be a prerequisite for the final steps of platelet production, proplatelet formation and platelet shedding. \textit{In vitro}, proplatelet formation was inhibited by Tpo in a dose-dependent manner [62,100,115,116].

Apart from its prominent effect on cells of the megakaryocytic lineage, Tpo has also been shown to be involved in the proliferation of primitive haematopoietic progenitor cells. Although the effect of Tpo alone is limited, \textit{in vitro} culture studies have shown that Tpo acts synergistically with early-acting cytokines in expanding long-term repopulating haematopoietic stem cells and in augmenting the production of committed progenitors [99,104,117-120]. More importantly, Tpo may be able to prevent the haematopoietic progenitor cell to undergo apoptosis and may play a role in the process of self-renewal of haematopoietic progenitor cells \textit{in vivo} [103,121,122]. In view of this feature, a lack of Tpo might explain why patients with congenital amegakaryocytic thrombocytopenia might develop aplastic anaemia a few years after birth in addition to their severe thrombocytopenia.

\textit{In vitro}, Tpo has been shown to be involved in platelet activation. At high concentrations, i.e. about 1000-fold above normal plasma levels, Tpo can directly induce platelet activation and aggregation \textit{in vitro} [123-125]. However, under pathological conditions, circulating Tpo levels are not expected to be more than a 100-fold increased. At this concentration, it was shown that Tpo has a priming effect on platelet responses to agonists such as ADP, thrombin and epinephrine [123,126-134]. In addition, it was shown that at physiological concentrations, Tpo can enhance \(a_{\text{IIb}}\beta_3\)-dependent platelet adhesion to fibrinogen, fibronectin and von Willebrand factor [135]. Improved \(a_{\text{IIb}}\beta_3\)-dependent adhesion mediated by Tpo has previously been shown for megakaryocytes, albeit with higher Tpo concentrations [136]. \textit{In vivo}, platelet activation and aggregation under the influence of Tpo might present a risk factor for thrombotic complications. By now, several clinical trials in which Tpo was administered to both healthy individuals and patients with thrombocytopenia have been completed [137]. No increased incidence of thrombotic events has been reported.

\subsection*{1.3.5.2 \textit{In vivo} effects}
Evidence for the important role of Tpo in megakaryocytopenesis was further confirmed by studies with knock-out mice lacking the Tpo gene [138,139] or the gene for the Tpo receptor, \textit{c-mpl} [88,139-142]. These mice have a more than 80-90\% reduction in their platelet counts, with low numbers of megakaryocytes with a low ploidy. The number of cells of all other haematopoietic cell types in these animals was unaffected, although the number of myeloid
committed progenitors was 50-60% reduced [139,140]. The regulators and/or mechanisms responsible for the residual platelet production in these animals are unknown. Double-mutant mice lacking both Mpl and IL-3, IL-6, IL-11 or LIF, have platelet counts comparable to the single-mutant Mpl-deficient mice, suggesting that as a single regulator, IL-3, IL-6, IL-11 or LIF are not involved in the residual platelet production seen in these mice [141,142]. In humans, mutations in the \textit{c-mpl} gene have been reported to underlie congenital amegakaryocytic thrombocytopenia (CAMT) [143,144]. Patients with CAMT have severe thrombocytopenia and lack megakaryocytes in their bone marrow. Bone-marrow cells from these patients did not respond to Tpo in \textit{in vitro} cultures [143-145], confirming the impaired expression or, in some cases, impaired function of Mpl.

Administration of recombinant Tpo or of PEG-rHuMGDF (polyethylene glycol-conjugated recombinant human megakaryocyte growth and development factor), a PEG-conjugated truncated non-glycosylated form of Tpo [146], to normal mice [41,42,87,147-150] non-human primates [151,152] or humans [153] has shown that Tpo enhances the thrombopoiesis; the number of circulating platelet increased in a dose-dependent manner without affecting the cell number of other cell lineages. For instance, Balb/c mice treated daily with recombinant Tpo showed a four-fold increase in platelet counts seven days after initiation of treatment [41,42]. Treatment of baboons with either rHuTpo or PEG-rHuMGDF resulted in a dose-dependent increase in platelet production, leading to peak levels in platelet count as high as 5 times baseline level, two weeks after initiation of Tpo administration [151,152]. In humans, a bolus injection of PEG-rHuMGDF of 3 µg/kg resulted in a two-fold increment in platelet counts on day 12 [137]. Platelet counts were back to normal again four weeks after PEG-rHuMGDF administration. Tpo administration to animals made thrombocytopenic either by myelosuppressive chemotherapy [87,154-157], irradiation [158-162] or both [147,163] has been shown to accelerate platelet recovery, improve platelet nadir and shorten the period of thrombocytopenia. Moreover, upon treatment with rHuTpo/PEG-rHuMGDF the recovery of haematopoietic progenitors in the bone marrow of all other lineages was also enhanced [87,147,158,160,161,164].

**Clinical trials**

Quite soon after the discovery of thrombopoietin, its potential application in the clinic was assessed. To date, the therapeutical potential of two recombinant Tpo preparations, PEG-rHuMGDF and rHuTpo, were evaluated in clinical studies. PEG-rHuMGDF is a truncated non-glycosylated form of Tpo that is PEG-conjugated to prolong its circulating half-life. It was initially developed by Amgen (Thousand Oaks, Ca, USA) and investigated in a partnership with Kirin Brewery Co. (Takasaki, Japan) and Zymogenetics Inc. (Seattle, WA, USA). rHuTpo was initially produced by Genentech and investigated in collaboration with Pharcmacia & Upjohn (Peapack, NJ, USA). In cancer patients, thrombocytopenia frequently occurs as a result of irradiation or chemotherapy. It was investigated whether administration of Tpo could accelerate platelet recovery and improve the platelet nadir in these patients. Dose-escalation studies were performed under different treatment schedules; i.e. thrombopoietin
was administered either before or after cytoreductive treatment or both, and for various periods of time, to establish safety and biological effects.

In all patients treated with Tpo in combination with moderate chemotherapy regimens, administration of Tpo was well tolerated and induced a dose-dependent increase in functionally normal platelets [146,164-168]. PEG-rHuMGDF [146,164,165,167,168] was administered subcutaneously for 10 days to patients with advanced cancer, whereas rHuTpo [166] was administered intravenously as a single bolus. When given to patients before moderate chemotherapy, platelet counts increased, but the length of the thrombocytopenic phase did not shorten [146,164-166]. When PEG-rHuMGDF was administered after moderate chemotherapy, platelet recovery was enhanced and platelet nadirs were improved [167,168]. Because the platelet transfusion requirement was low in these studies, it could not be established whether treatment with Tpo reduced the number of required platelet transfusions.

An indication that Tpo treatment can reduce the need for platelet transfusions came from a phase I/II trial in which 29 patients with gynaecological cancer were enrolled [169]. In this study, the patients were repeatedly treated with high-dose chemotherapy and received rHuTpo in a dose-escalation phase to determine safety and the optimal biological dose and in a dose-expansion phase to assess safety and optimal biological activity. In the dose-escalation phase (n=16), a single dose of rHuTpo was administered three weeks before the first cycle of chemotherapy with carboplatin. Three weeks after cycle 1, when platelet and neutrophil count were recovered, patients were treated for the second time with carboplatin, followed by four doses rHuTpo every other day (cycle 2). Treatment with rHuTpo was well tolerated and resulted in an enhanced platelet recovery (platelet count > 50x10^9/L: 6 days vs. 3 days) and an ameliorated platelet nadir (35x10^9 platelets/L vs. 53x10^9 platelets/L) when comparing cycle 2 with cycle 1. In the dose expansion phase (n=12), patients were treated with the optimal dosing schedule of 1.2 µg/kg rHuTpo after cycle 2. Again platelet nadir was improved and platelet recovery enhanced. Moreover, the need for platelet transfusion was reduced with 50%-75% of the patients required platelet transfusions after cycle 1 whereas 25% of the patients needed platelet transfusions after cycle 2.

Platelet transfusion requirements were also reduced in a study of patients with breast cancer who were treated with high-dose chemotherapy in combination with autologous bone-marrow transplantation (ABMT). PEG-rHuMGDF was administered on day 0 (=day of ABMT) up to the day when platelet counts were higher than 250x10^9/L. Platelet recovery was accelerated and the number of platelet transfusions were reduced from 11 in the placebo group to 8 and 6 in the PEG-rHuMGDF groups (5 µg/kg/day and 10 µg/kg/day respectively) [170].

The efficacy of recombinant Tpo administration in combination with high-dose chemotherapy with peripheral blood progenitor support is limited. Administration of PEG-rHuMGDF either before or after chemotherapy with peripheral-blood progenitor support was not effective in enhancing platelet recovery or in reducing the number of platelet transfusions [171,172]. Despite the capacity of recombinant Tpo to mobilise haematopoietic stem cells and progenitors to the peripheral blood [164,166,167,173], cell maturation is not enhanced. This was to be expected. In contrast to the effect of granulocyte colony-stimulating factor (G-CSF) on
neutrophil granulocytopenosis, which shortens the generation time of neutrophils [174], Tpo does shorten the generation time of platelets. Therefore, *ex vivo* expansion of progenitor cells with recombinant Tpo before returning the cells to the patients might be more promising in accelerating platelet recovery. Bertolini et al. [107] have shown in a feasibility study in which 10 patients with cancer were enrolled a favourable effect of reinfusion of *ex vivo* expanded autologous megakaryocyte progenitor cells in combination with unmanipulated cells. Autologous CD34+ cells were expanded in serum-free medium in the presence of MGDF, stem cell factor (SCF), IL-3, IL-6, IL-11, Flt3-ligand and macrophage inflammatory protein-1α. Upon reinfusion of the megakaryocytic progenitors, eight patients needed a single platelet transfusion, whereas two patients, receiving the highest doses of cultured megakaryocytic progenitors, did not require a platelet transfusion. This compared favourably with a historic control group of 14 patients with an average platelet transfusion need of 1.2. Further studies to optimise culture and reinfusion conditions are ongoing.

Administration of PEG-rHuMGDF was not effective when given to patients with acute myeloid leukaemia after induction therapy [175,176]. Encouraging results, however, have been reported in an abstract on a trial in which PEG-rHuMGDF was administered to HIV patients to correct platelet count (Fig 4). Platelet counts increased 10-fold within 14 days and returned to baseline levels 2 weeks after termination of treatment. Apoptosis of megakaryocytes was increased in pre-treatment samples but normalised during treatment [153]. This suggests that Tpo increases the effectiveness of platelet production by suppressing apoptosis. In addition, increased production of megakaryocytes under the influence of Tpo might play a role.

In all studies, rHuTpo and PEG-rHuMGDF proved to be safe and well tolerated. However, in 10% of the healthy volunteers that received multiple doses of PEG-rHuTpo subcutaneously, neutralising antibodies were formed that cross-reacted with endogenous Tpo, resulting in thrombocytopenia. Also, three cancer patients involved in a clinical trial with PEG-rHuMGDF were reported to have developed neutralising antibodies [153,177-180]. As a result of this, all clinical trials with PEG-rHuMGDF have been stopped. No such problems have
been reported with rHuTpo and the development of this preparation still continues. Thrombopoietin mimetic peptides, which are functionally similar to Tpo but bear no sequence homology with Tpo, might represent an alternative for recombinant thrombopoietin preparations. Two such peptides have been described, both of which have been shown to stimulate platelet production in animal models [181-184]. Its clinical potential in humans remains to be investigated.

Neutralising antibodies against Tpo have been described, independent of Tpo treatment, in a 70-year old patient with amegakaryocytic thrombocytopenic purpura (AMTP) [185]. AMTP is a rare disease that is characterised by severe thrombocytopenia associated with a lack of megakaryocytes in the bone marrow. Treatment of this patient with cyclosporin A resulted in remission, suggesting that the thrombocytopenia was caused by an immune-mediated mechanism. This strongly indicates that the Tpo autoantibodies were involved in the pathogenesis of this patient [185].

1.4 The Tpo-receptor, Mpl

1.4.1 Discovery and structural characteristics

As mentioned in paragraph 1.3, the identification of the murine retrovirus, myeloproliferative leukaemia virus (MPLV) [36], followed by the cloning of the viral oncogene v-mpl [37] and its cellular homologue c-mpl [38] was the key to the discovery of Tpo. The products of c-mpl were shown to be members of the haematopoietin receptor superfamily [186,187]. The extracellular domain of the products contain two copies of the haematopoietin receptor domain, the WSXWS box, and displays four conserved cysteine residues at the N-terminal domain (Figure 6). No consensus sequence for protein kinase activity was detected in the cytoplasmic domain. These three features are characteristic for members of the haematopoietin receptor su-
perfamily [188,189]. In humans, the c-mpl gene is located on the short arm of chromosome 1 (1p34), spans approximately 17 kb and contains 12 exons [190,191] (Figure 6). Its genomic organisation is similar to that found in other genes coding for cytokine receptors, i.e. the IL-3, GM-CSF and IL-5 receptor [192]. As a result of alternative splicing, four Mpl mRNA isoforms are formed, Mpl-P, Mpl-K, Mpl-S and Mpl-del [38,191,193,194]. Mpl-P encodes the full-length functional Tpo receptor of 635 amino acids, with an extracellular domain of 463 amino acids, a transmembrane domain of 22 amino acids and a cytoplasmic domain of 122 amino acids and an estimated mass of 71 kDa. In addition to the two WSXWS boxes, the extracellular domain contains several potential N-glycosylation sites. Mpl-K encodes a shorter Mpl isoform (572 amino acids; 65 kDa) that has an extracellular and transmembrane domain similar to Mpl-P, but contains a different cytoplasmatic signaling domain, as a result of a premature termination of the transcript at intron 10. PCR analysis of several hematopoietic cell lines has shown that Mpl-P and -K are always coexpressed [38]. Mpl-S encodes a putative soluble form of the Mpl receptor, because the mRNA lacks exon 9 and 10. So far, no studies on Tpo binding or signaling via Mpl-K or Mpl-S have been published. The Mpl-del isoform has been recently identified [194]. It lacks 72 bp in the extracellular region as a result of alternative RNA splicing between exon 8 and 9. Although the protein is expressed, it is not incorporated into the cell membrane. It might play a role in modulating the expression of functional Mpl.

1.4.2 Expression of the Tpo-receptor, Mpl

Immunofluorescent analysis has shown that Mpl is expressed on a subset of early progenitor cells and on all cells from the megakaryocytic lineage, including platelets [122,195]. Seventy percent of the human CD34+/CD38- cells, a subset that is highly enriched in stem cell progenitors, expresses Mpl. This CD34+/CD38+/Mpl+ subpopulation has been shown to engraft SCID mice more efficiently than the Mpl negative population [122]. Platelets express a small number of Mpl molecules, approximately 30-60 per platelet, with a high binding-affinity for Tpo of about 200 pmol/L [13-15]. On megakaryocytes, the number of Tpo receptors was reported to vary from approximately 200 on cord blood (CB)-derived megakaryocytes (after culture of CB mononuclear cells) to 2000 on peripheral blood (PB)-derived megakaryocytes (after culture of CD34+ PB cells) [196]. In humans, transcripts of the Tpo receptor, Mpl, are mainly detected in hematopoietic tissues, i.e. bone marrow, foetal liver and spleen, but also in the placenta, in the endothelium and in the brain [38,39,197]. A large panel of solid tumour cell lines was found negative for expression of Mpl RNA transcripts, with the exception of the hepatocellular carcinoma cell line Hep3B [197,198]. No Mpl mRNA transcripts were found in several T-, B- and, NK-cell lines [38,39,197,198]. Of the cell lines derived from human hematopoietic malignancies, the majority of megakaryocytic cell lines were found positive, as were some erythroleukemic lines. In about 50% of the patients with acute myeloid leukaemia, blast cells express Mpl. In AML-M7, representing malignant expansion of megakaryoblastic cells (according to the French-American-British classification of AML), Mpl expression is always positive [199-203]. Other AML subtypes show variable expression of Mpl [199-206]. Coexpression of both Tpo and Mpl transcripts have also been reported in AML, especially in

24
M7 cases [200]. In myelodysplasia, Mpl expression has been reported in chronic myelomonocytic leukaemia cells [207] and in cases of refractory anaemia with excess blasts (RAEB) and RAEB in transformation (RAEBt), but not in refractory anaemia (RA) or refractory anaemia with ringed sideroblasts (RARS) [204,207,208]. In both AML and RAEB and RAEBt, but not in CML, Mpl expression correlates with an unfavourable prognosis. Several in vitro studies have shown that blast cells expressing Mpl, proliferate in response to Tpo [199,199,201,203,206,208-213]. Taken together, these results suggest that Tpo and its receptor may be involved in the abnormal proliferation and differentiation in AML and MDS. In AML-M7, the coexpression of both Tpo and its receptor might result in autostimulation.

1.4.3 Signal transduction

The Tpo molecule has two receptor-binding domains [214,215]. Binding of Tpo to its receptor Mpl is thought to result in homodimerization, which leads to downstream signal transduction [187,216]. Signal transduction has been studied in cell lines [217-224,224-227], purified platelets [133,228,229] and megakaryocytes [224,230]. It involves activation of the JAK-STAT pathway, including phosphorylation of Jak2 [133,217-220,226-230] and TYK2 [133,218,229,230] followed by phosphorylation of the signal-transducing molecules STAT1 [218,222,227], STAT3 [133,223,226,227,229-231] and STAT5 [217,218,223,226,229-231]. In addition, activation of the Ras-MAPK signaling pathway was reported [220-222,224,225,227,228,232] as well as phosphorylation of the receptor itself [220]. The cytoplasmic domain of Mpl comprises 122 amino acids and can be divided in at least two functional elements. The membrane-proximal subdomain includes two structural motifs, box 1 and box 2, which are conserved among members of the haematopoietic receptor superfamily [186,233]. Deletion and mutational analysis has shown that these motifs are crucial for cell proliferation and maturation and are associated with JAK2 activation [227,233-239]. Activation of JAK2 seems to be essential for signaling. Absence of the JAK2 gene in mice is lethal due to the absence of definitive erythrocytopoiesis. In addition, the haematopoietic progenitors from the foetal liver of these mice do not respond to Tpo [240]. Apart from JAK2, TYK2 has also been shown to be phosphorylated in response to Tpo. However, the contribution of TYK2 in the signaling cascade seems to be less important. Analysis of c-mpl transfected sarcoma cells lacking either JAK2 or TYK2 expression has shown that Tpo-induced signaling is intact in TYK2-deficient cells but absent in JAK2-deficient cells [241].

The carboxy-terminal subdomain of the cytoplasmic intracellular domain is involved in differentiation [234-237]. Its presence is not necessary for proliferation [234,242]. The signaling pathways associated with the C-terminal domain seem to involve the Ras-MAPK cascade [222,225,227,243]. The exact contribution of the different signaling pathways to the induction of various cellular processes and its relation with different receptor domains still remains to be elucidated.
Figure 7: Basic regulation of Tpo levels. Tpo is constitutively produced by the liver and the kidney and reaches the circulation. In the bone marrow, Tpo binds to megakaryocytic progenitors and stimulates platelet production. Platelets come into the circulation and bind Tpo, thus regulating their own production level. Finally, platelets are destroyed in the spleen.

1.5 Controlling thrombocytopoiesis by regulation of thrombopoietin levels

With Tpo being the main physiological regulator of platelet production, it was expected that Tpo levels vary inversely with the need for thrombocytes. Previously, it had been shown that the thrombopoietic capacity of plasma from thrombocytopenic animals is increased suggesting that Tpo levels are regulated at some level. Initially, a model was proposed that assumes a fixed Tpo production and regulation of Tpo levels by Mpl-mediated Tpo uptake. Thus, the total mass of megakaryocytes and platelets, which both express Mpl, determines the amount of circulating Tpo. When platelet counts fall, Tpo levels will rise and stimulate megakaryocytopoiesis. Alternatively, upregulation of Tpo production at the transcriptional level may occur. These two models are not mutually exclusive. Increasing evidence suggests that both models apply.

1.5.1 Fixed Tpo production and c-Mpl-mediated Tpo uptake

This model was first postulated by Kuter and Rosenberg [244]. Already before the actual cloning of thrombopoietin, these investigators reported that the concentration of a factor responsible for thrombocytopoiesis, designated megapoietin, is increased in thrombocytopenic rats and rabbits and is decreased during thrombocytosis [245,246]. Its concentration is inversely and proportionally related to platelet count. Increased levels persist for as long as the stimulus, i.e. thrombocytopenia, is present. The appearance of this factor in the circulation occurs a few hours after the induction of thrombocytopenia and is maximal after 24 hours [246]. After the isolation of Tpo, Kuter and Rosenberg showed that megapoietin was identical to Tpo [244]. In a model in which rabbits were made thrombocytopenic through busulphan treatment, they showed that Tpo levels decreased after platelet transfusion. In addition, platelets were found to remove Tpo from plasma in a temperature-dependent fashion [244]. Based
on these findings, the investigators postulated that Tpo production occurs at a constant rate and that platelets are responsible for the clearance of Tpo from the circulation (Figure 7). In this way, platelets modulate Tpo levels and ultimately regulate their own production [246]. This mechanism, in which the terminally differentiated cell regulates the concentration of its own regulatory factor, is not uncommon. For instance, the same mechanism applies for the terminally differentiated macrophage, which metabolises M-CSF [247,248], and the neutrophil, which has been reported to metabolise G-CSF [249-251]. Evidence in favour of this mechanism for Tpo came from both in vitro and in vivo studies.

1.5.1.1 Platelets bind and metabolise Tpo
Several studies demonstrated that platelets bind and metabolise Tpo. In vitro studies have shown that this occurs in a specific, dose, time and temperature-dependent manner [14-17]. In vivo, complementary to the transfusion studies of Kuter and Rosenberg [244], it was demonstrated that Tpo levels decreases after platelet transfusion in both animal models [17] and human subjects (see also Chapter 2&3) [252-254]. Injection of radiolabeled Tpo to various mouse strains provided further evidence that platelets bind Tpo. Platelets from c-mpl knockout mice (mpl−/−), are unable to bind and metabolise Tpo, and Tpo half-life in mpl−/− mice is prolonged compared to that in normal mice [17].

1.5.1.2 Megakaryocytes participate in Tpo clearance
Although the above-mentioned studies confirmed the central role of platelets in clearance of Tpo from the circulation, platelets appeared to be not the sole contributor to this mechanism. In addition to platelets, megakaryocytes also seem to be involved in regulation of Tpo levels via Mpl-mediated Tpo binding. This is analogous to the binding of Epo, the growth factor regulating red blood cell production, by red blood cell precursors in the bone marrow [255]. In NF-E2 knockout mice, the absence of the erythroid transcription factor NF-E2 results in a late arrest of megakaryocytic maturation and profound thrombocytopenia [256,257]. Instead of the expected increase in thrombopoietin as a result of a reduced binding to platelets, Tpo levels were normal [257]. Tpo mRNA levels in the foetal liver of NF-E2 knockouts were not different from that in control animals, suggesting that Tpo production was not impaired. Tracing of radiolabeled Tpo after intravenous administration in NF-E2−/− mice, has shown that Tpo is associated with haematopoietic tissues. In particular, the radiolabeled Tpo was almost exclusively found associated with megakaryocytes and small abnormal platelet-like particles. Assuming that these particles have a lower Tpo binding capacity than platelets, this model provides evidence that megakaryocytes are involved in clearance of Tpo from the circulation. Evaluation of the relation between platelet counts and Tpo levels during chemotherapy provided further evidence. Numerous groups have reported that platelet counts and Tpo levels are inversely related during chemotherapy, i.e. when platelet counts decrease after chemotherapy, Tpo levels rise and vice versa (see also Chapter 2) [96,254,258-264]. Detailed cross-correlation analysis of this relation, however, has shown that the Tpo response precedes the platelet response by about one day [265]. Similar findings of declining Tpo levels before the increase in platelet counts were also reported in a case of cyclic thrombocytopenia [266,267].
A model in which only platelets are responsible for Tpo clearance does not explain these observations. Inclusion of megakaryocytes does.

1.5.1.3 Tpo production is constitutive: evidence against transcriptional regulation

As already mentioned in paragraph 1.3.4, no transcriptional regulation of the Tpo gene in the liver and the spleen has been reported. Upon induction of thrombocytopenia in mice, Tpo mRNA levels in these organs are not increased [16,17,65,85,86], nor is there any difference in the generation of alternative Tpo mRNA splice forms [16]. In addition, in thrombocytopenic animals (i.e. with highly elevated platelet counts), Tpo mRNA levels remain unchanged as well.[85] In support of the notion that Tpo transcription is not regulated are the findings with respect to the Tpo and Mpl knockout mice [88,138]. In the Tpo knockout mice, platelet and megakaryocyte counts are decreased with more than 80%. In heterozygous mice this decrease is 50%. This gene dosage effect is in line with a constitutive expression of Tpo [138]. In analogy, no upregulation of Tpo mRNA is seen in Mpl knockout animals despite an 85% decrease in megakaryocyte and platelet mass [88].

1.5.2 Evidence in favour of transcriptional regulation and other forms of Tpo regulation

In analogy with the transcriptional regulation of the Epo gene, it has been proposed that Tpo levels might also be regulated at the level of gene expression. In case of Epo, a sensing mechanism in the kidney detects alterations in the oxygen pressure and varies the transcription of the Epo gene accordingly [268]. Although a different "sensing mechanism" would be needed for Tpo, transcriptional regulation might play a role to some extent. For instance, upregulation of Tpo mRNA by bone-marrow stromal cells in response to thrombocytopenia has been described [61,63] (see also §1.3.4). In addition, there are indications that inflammatory cytokines such as IL-6 might induce upregulation of Tpo transcription. IL-6 has been shown to increase the Tpo production by hepatoblastoma cell lines [269] (and Chapter 8)

Three other potential mechanisms might influence the available amount of biologically active Tpo. First, it has been reported that incubation of Tpo with thrombin results in proteolytic cleavage of Tpo [270]. Some of the generated Tpo fragments have enhanced biological activity in vitro [270]. Possibly, in vivo, this mechanism might modulate Tpo activity at a local level. Certain Tpo products might be over- or under-represented in haematological disorders. So far, characterisation of native thrombopoietin forms obtained from plasma of healthy individuals or from individuals with haematological disorders, such as idiopathic thrombocytopenic purpura, aplastic anaemia, essential thrombocythaemia, polycythaemia vera and disseminated intravascular coagulation, have revealed no differences in size distribution [50]. In all cases, Tpo is predominantly present in the full-length form, although a small amount of truncated forms were also detected.

Second, release of Tpo by activated platelets, such as described in more detail in Chapter 9 [271] might influence the available amount of biologically active Tpo.

Third, Tpo production might be modulated at the translational level. It has been reported that translation of Tpo mRNA under normal conditions is almost completely inhibited by the presence of an AUG codon in the 5'-untranslated region [272]. The importance of this mechanism
became apparent by the finding that in four families with hereditary thrombocytosis, this inhibition was lost, resulting in Tpo overproduction [273-276].

1.6 Measurement of Tpo levels as a diagnostic tool

After the discovery of thrombopoietin, several assays have been developed to measure the concentration of this cytokine. Initially, bioassays were used in which the Tpo concentration was related to the extent of the proliferation of Tpo-dependent cell lines. The murine cell lines Ba/F3 and 32D were transfected with c-mpl for this purpose and thereby became dependent for their growth on Tpo [40,41,43-45] However, bioassays are time consuming and not very sensitive. In addition, for diagnostic purposes, bioassays are not always reliable because toxic substances in blood might affect cell growth. Immunoassays provide a good alternative. To date, five different immunoassays have been developed, all of which are based on an enzyme-linked immunosorbent assay format (see also Chapter 2) [254,259,277-279]. Measurement of Tpo in patients with thrombocytopenia has shown that the level of Tpo is indicative for the underlying cause of thrombocytopenia. High levels (5 to 100 times normal) have been reported when thrombocytopenia results from a decreased Tpo production, such as occurs in amegakaryocytic thrombocytopenia, aplastic anaemia or after myelosuppressive therapy (see also Chapter 2, 5 & 6) [144,254,259,260,262-265,267,277,280-282]. Normal or slightly elevated levels are found when thrombocytopenia is caused by platelet destruction in the periphery, such as occurs in idiopathic thrombocytopenic purpura (see also Chapter 5 & 6) [260,280-286]. Therefore, measurement of circulating Tpo can be used as an aid in the classification of thrombocytopenia. Tpo levels have been assessed in various other patient groups and the gathered data were analysed with respect to their diagnostic potential and used to get insight into the regulation of thrombocytopoiesis under the influence of Tpo. In the following chapters, our data and the findings from other investigators will be discussed in more detail.

1.7 Outline of this thesis

The work described in this thesis focuses on the diagnostic value of thrombopoietin level measurements in blood samples from different patient groups. These studies add to the understanding of the mechanisms underlying the regulation of thrombopoietin levels in the circulation. In addition, the interplay between Tpo and platelets was assessed. To measure Tpo concentrations, a sandwich ELISA was developed, which is described in Chapter 2. Serial measurements of the plasma Tpo concentration and platelet-associated Tpo in patients who received platelet transfusions showed that platelets can bind Tpo, and thus remove it from the circulation (Chapter 3). Although Tpo mRNA was found in both liver and kidney tissue, the production of Tpo in vivo seemed to involve mainly the liver, since Tpo levels in patients with chronic renal failure and normal platelet counts were within the normal range (Chapter 4). In Chapter 5 and 6, the plasma Tpo levels in different groups of patients with thrombocytopenia are described. It was shown that measurement of Tpo in these patients is a helpful tool in distinguishing thrombocytopenia as a result of increased platelet destruction (high Tpo levels) from decreased platelet production (normal Tpo levels). Patient groups
Chapter 1

with abnormally high platelet counts were analysed in Chapter 7. In these patients, relatively high Tpo levels were found. Despite the presence of a high platelet number, Tpo levels were within the normal range or even slightly increased, suggesting improper downregulation of the Tpo concentration. Differences in platelet Tpo content in patients with reactive thrombocythaemia versus primary thrombocythaemia suggest a difference in the mechanism underlying the relatively high Tpo level. In patients undergoing major surgery, an increase in Tpo levels was found to precede an increase in platelet count (Chapter 8). The origin of this Tpo increase is not fully known. Possibly, inflammatory factors that increase upon surgery, such as IL-6 (which was found to be increased), are instrumental in increasing the Tpo production by the liver (Chapter 8). Platelets can also release Tpo upon activation as described in Chapter 9. In vivo, this mechanism might play a role in patients with disseminated intravascular coagulation (Chapter 9) and endotoxiaemia (Chapter 10). In these patients, elevated Tpo levels were found. Apart from Tpo release, here as well, inflammatory cytokines, such as IL-6, might enhance Tpo production.

Analysis of the patient groups mentioned above showed that increased Tpo levels were only found in association with thrombocytopenia, either due to decreased platelet production or to increased platelet consumption in the periphery, or preceding an elevation in platelet counts such as in the patients undergoing major surgery. In Chapter 11, elevated Tpo levels are reported in patients with multiple myeloma in association with normal platelet counts. As the overall haematopoiesis is suppressed in these patients due to malignant bone marrow infiltration, the elevated Tpo levels suggest that Tpo is involved in the maintenance of platelet production. Multiple myeloma cells might be either directly, or indirectly, involved in the production of thrombopoietin. In the last chapters, all results are summarised and the data are discussed with respect to the diagnostic value of Tpo measurements and the implications for the regulation of Tpo levels in the circulation.

1.8 References


General introduction


Chapter 1


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Chapter 1


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Chapter 1


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Chapter 1


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