CHAPTER I

Introduction
MYELODYSPLASTIC SYNDROMES

Myelodysplastic syndromes (MDS) are a heterogeneous group of potentially malignant clonal stem cell disorders characterized by an ineffective and dysplastic hematopoiesis, which frequently lead to fatal cytopenias or acute myeloid leukemia (AML). MDS demonstrate a wide range of clinical characteristics, cytological and pathological features, and cytogenetic abnormalities. Multiple classification and prognostic scoring systems have been proposed to facilitate medically appropriate treatment and management decisions for MDS patients. By means of morphology and cytochemical criteria MDS were originally divided into five subgroups (French-American-British (FAB) classification) (Table 1). Through the years it was demonstrated that the FAB classification has clear prognostic impact, distinguishing (i) a lower-risk group comprising of refractory anemia (RA) and RA with ringed sideroblasts (RARS) with median survival of approximately 4-5 years and an AML transformation rate of <25%, (ii) an intermediate-risk group with a survival of about 1 year including RA with excess blasts (RAEB), and (iii) a higher-risk group which develops AML or die within a few months, consisting of RAEB in transformation (RAEB-t). The prognosis for chronic myelomonocytic leukemia (CMML) is variable and depends on the bone marrow blast count and whether myelodysplastic or myeloproliferative features predominate. Recently, a classification from the World Health Organization (WHO) was introduced, with the purpose to refine the FAB classification by incorporating additional diagnostic information, such as cytogenetics (Table 1). Although controversies remain, the WHO revisions have been shown to provide more homogeneous subgroups of MDS patients and greater prognostic importance than the FAB classification. In particular, the number of lineage dysplasia, the bone marrow blast count and the karyotype appear to be the most important prognostic parameters in MDS. Two of these factors, bone marrow blast percentage and karyotype, in combination with the number of cytopenias were the basis for the International Prognostic Scoring System (IPSS), introduced in 1997 (Table 2 and 3). The IPSS reliably estimates survival and risk of AML transformation for MDS patients and is universally accepted. The IPSS low-risk group is primarily found in the lower-risk FAB subgroups (RA and RARS). RA and RARS are therefore generally considered as low-risk MDS. Other definitions of low-risk MDS are IPSS categories low- and intermediate-1 risk, while the intermediate-2 and high-risk categories are designated as high-risk MDS. The distinction between low- and high-risk MDS is also important from a pathophysiological point of view. In low-risk MDS it is reported that
<table>
<thead>
<tr>
<th>FAB</th>
<th>Distribution, %</th>
<th>Blast in BM, %</th>
<th>Ringed sideroblasts, %</th>
<th>Median survival, months</th>
<th>WHO</th>
<th>Distribution, %</th>
<th>Blast in BM, %</th>
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<th>Median survival, months</th>
<th>Progression to AML, %</th>
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<tr>
<td>RA</td>
<td>36</td>
<td>&lt;5</td>
<td>&lt;15</td>
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<td>RCMD</td>
<td>25-30</td>
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<td>35</td>
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<tr>
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<td>15</td>
<td>&lt;20</td>
<td>NA</td>
<td>12</td>
<td>Excluded*</td>
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**Abbreviations:** AML, acute myeloid leukemia; BM, bone marrow; CMML, chronic myelomonocytic leukemia; FAB, French-American-British; MDS, myeloid dysplastic syndromes; MDS-U, MDS-unclassified; NA, not applicable; RA, refractory anemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; RAEB-t, RAEB in transformation; RCMD, refractory cytopenias with multiple lineage dysplasia; RCMD-RS, RCMD with ringed sideroblasts; WHO, World Health Organisation. * placed in the newly created category “myelodysplastic/myeloproliferative diseases”
enhanced apoptosis of terminally differentiated marrow cells plays an important role in the pathogenesis of MDS, in particular in explaining the paradox between a hypercellular bone marrow and peripheral blood cytopenias. In high-risk MDS, cell proliferation and survival prevails over apoptosis, explaining why progression to AML is common in this group.\(^3\) There is a large amount of \textit{in vitro} evidence in favor of these disease mechanisms.\(^5\) There are however also controversies, for example regarding the degree and the clinical relevance of the intramedullary apoptosis.\(^6\) Furthermore, most experiments have been done on the myeloid and erythroid lineage. Data on megakaryopoiesis, platelet production, and mechanisms of thrombocytopenia in MDS are scarce.

Previous studies have suggested that apart from a decreased production of platelets, an increased platelet destruction might also contribute to thrombocytopenia in (some) MDS patients, suggesting an immune-mediated process.\(^7\)
CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA

Idiopathic or immune thrombocytopenic purpura (ITP) is characterized by an isolated thrombocytopenia in an otherwise healthy person. A generally accepted etiology of ITP is that it is caused by platelet autoantibody-mediated increased destruction of platelets, especially in the spleen. Evidence for this autoantibody-mediated thrombocytopenia came from experiments in the early 1950’s in which infusions of plasma from ITP patients induced thrombocytopenia in healthy volunteers. Autoantibodies against platelet membrane glycoproteins (GP) IIb/IIIa and Ib/IX are present in 50-75% of ITP patients, which are predominantly IgG. Besides antibody-mediated platelet destruction, cytotoxic T cell-mediated lysis of platelets has recently been demonstrated in vitro using CD3+/CD8+ lymphocytes from patients with active ITP. In the classical view of ITP enhanced peripheral destruction of platelets leads to a compensatory increase in platelet production. Findings of increased numbers of megakaryocytes in bone marrow samples of ITP patients and normal plasma or serum thrombopoietin (TPO) concentrations, are often considered to support this notion. However, there is also evidence that megakaryopoiesis and platelet production can be suppressed in ITP. Multiple studies have demonstrated that in ITP i) antiplatelet autoantibodies can also recognize megakaryocytes; ii) megakaryocytes are morphologically abnormal, and relatively more immature; and iii) the platelet turnover, also termed the platelet production rate, which is estimated by radiolabeled platelet studies, is suppressed in large subgroups of patients. Furthermore, recent experiments by other groups that have been published during the progress of this thesis have shown that megakaryopoiesis can be suppressed in vitro by antiplatelet antibodies in approximately two-third of ITP patients. ITP therefore appears to be a heterogeneous disease, in which both peripheral platelet destruction and impaired platelet production contribute to thrombocytopenia in a varying degree. Differential expression of antigens or epitopes on platelets and megakaryocytes, the type and titer of the antiplatelet antibody, the difference in the capacity and function of reticuloendothelial system and the extent of T-lymphocyte-mediated cytotoxicity might all be important in explaining these interindividual variations in disturbed platelet destruction and production.

Thus, although ITP and MDS are evidently totally different diseases, there are also similarities with regard to clinical presentation and certain pathophysiological mechanisms, such as increased platelet destruction and decreased platelet production. There-
fore, in this thesis both ITP and MDS patients were studied regarding abnormalities in megakaryopoiesis, platelet production and platelet survival. These processes can be studied in vivo and in vitro with several techniques, including electron microscopy, platelet kinetic studies, plasma TPO and glycocalicin measurements, and immunohistochemistry for assessment of cell death in megakaryocytes.

TECHNIQUES TO STUDY MEGAKARYOCYTES AND PLATELETS

Electron microscopy
The megakaryocyte is the largest cell in the bone marrow. Therefore, megakaryocytes are easily identified by scanning a bone marrow smear with light microscopy at low power. Megakaryocytes were among the earliest hematopoietic cells described with considerable accuracy. Megakaryocytes are usually more numerous in bone marrow particles, and the number in bone marrow smears varies depending on the cellularity of the specimen. Megakaryocytes make up about 0.03 to 0.5% of the nucleated cells in human bone marrow. The number of megakaryocytes has been estimated by relating megakaryocytes to the erythroid precursor mass determined from plasma iron turnover measurements. Using this method, the number of megakaryocytes in humans is estimated at $6.1 \pm 0.7 \times 10^6$/kg.

Methods to study megakaryocytes have been hampered by both their low frequency in the bone marrow and their vulnerability. Since it has become possible to culture megakaryocytes in vitro, the knowledge regarding megakaryocyte differentiation, maturation and platelet formation has considerably increased. Most information about megakaryocyte morphology has been obtained by studying the ultrastructure of human and rodent (murine/rat) bone marrow.

Electron microscopy (EM) has a magnifying potential to distinguish individual molecules. Analysis with EM can reveal important information concerning the structure and function of cells and cellular organelles. There are two types of EM techniques: transmission and scanning. The latter form is irrelevant for the scope of this thesis. In transmission EM high speed electrons are focused on an ultrathin section. As the electrons pass through the section, different parts of the cell absorb, diffract, or transmit the electron beam. Objects can be magnified as many as one million times, structures as small as 0.2 to 0.3 nm can be resolved.

Ultrastructurally, three stages of megakaryocyte maturation can be recognized. The
stage I (immature) megakaryocyte or megakaryoblast comprises about 19% of the megakaryocyte population and is 6-24 µm in diameter. It has a large oval, indented or lobed nucleus with several prominent nucleoli, and endomitosis can be observed. The basophilia of the cytoplasm on light microscopy reflects the abundance of ribosomes (for protein synthesis) seen ultrastructurally. The megakaryoblast contains a few mitochondria, and a few or no α-granules and only rudiments of the nascent demarcation membrane system (DMS). Stage II (maturing) megakaryocytes or promegakaryocytes are 14-30 µm in diameter and make up about 25% of the megakaryocyte population. In this stage endomitosis is completed. Polyploidy ranges from 8N to 64N. Granules are developing, especially in the perinuclear zone. Mature or stage III megakaryocytes make up about 56% of the megakaryocyte population and are 40 to 56 µm in diameter. Due to the decrease in ribosomes and the increase in granules the cytoplasm is more acidophilic. Mature megakaryocytes contain a well developed DMS.25,26

The DMS is a smooth membrane system that stands in continuity with the external milieu. Platelet glycoproteins, such as GPIIb-IIIa, are expressed on both the plasma membrane and the DMS of maturing cells. The DMS plays an important role in platelet formation, but probably not by compartmentalizing (“demarcating”) the megakaryocyte cytoplasm into platelet territories. Instead, the DMS evaginates and forms processes or proplatelets, which subsequently give rise to platelets. DMS is therefore called a “misnomer”, and the name “invaginated membrane system” has been proposed as being more appropriate.27

EM studies in ITP are scarce and have shown conflicting results, ranging from megakaryocytes showing practically normal morphology28,29 to abundant abnormalities.16 Abnormal megakaryocyte ultrastructure, including vacuoles, markedly distended DMS, mitochondrial swelling, and emperipolesis (active penetration by one cell into and through a larger cell) of other marrow cells, has been reported. The abnormalities are usually ascribed to a damaging effect of antiplatelet autoantibodies.30 Some authors have observed abnormalities only in megakaryocytes which are in the stage of platelet formation. This has been explained by the notion, that in this stage the DMS has reached continuity with the plasma membrane of the megakaryocyte and antibodies might be able to react with GP which are otherwise hidden in the interior of the megakaryocyte. In ITP megakaryocytes with normal ultrastructure platelet territories had not yet reached the peripheral zone.16

In MDS, ultrastructural studies of megakaryocytes are also sparse. Abnormalities such as scanty heterochromatin, numerous cytoplasmic microvacuoles, and a shift towards immaturity have been described.31

An additional reason to use EM in our studies is the fact that EM is the most reli-
able technique to detect and visualize cell death. Many forms of cell death, including apoptosis, autophagic cell death and necrosis are essentially defined by morphology, especially by ultrastructural characteristics. Although biochemical characteristics of cell death pathways are increasingly discovered, the most important classifications of cell death are also still based on morphology. Limitations of EM as a cell death detecting method are that predominantly late stages of cell death are detected. Experts in the field therefore advise to use additional techniques for cell death assessment simultaneously. For more extensive information on this topic the reader is referred to Chapter II.

**Platelet kinetic studies**

By studying the fate of intravenously injected *in vitro* radiolabeled autologous platelets in the *in vivo* situation several platelet kinetic parameters can be determined: platelet life span (mean platelet life, MPL), platelet distribution (initial platelet recovery and organ uptake) and the platelet turnover rate. Platelet turnover reflects platelet removal from the circulation. In the steady state platelet turnover equals platelet release from the bone marrow into the circulation, and an estimate of the number of platelets removed from and replenished to the circulation each day can be obtained by dividing the circulating platelet count by the survival time in days. When appropriate corrections are made for variations in the initial platelet recovery, a value for platelet turnover rate, also called platelet production rate (PPR) can be obtained. In other words, PPR can also be defined as the number of platelets entering the circulation to maintain the platelet count. Since the PPR represents release of platelets to the peripheral blood, it is not necessarily identical to the real platelet production. Harker and Finch originally defined PPR as the “effective platelet production” and the megakaryocyte mass, the product of the mean megakaryocyte volume and total megakaryocyte number, as a reflection of the platelet producing capacity of the bone marrow, and therefore as the “total platelet production”. A disparity between these two parameters is believed to represent “ineffective thrombopoiesis”.

Platelet kinetic studies have been used to differentiate thrombocytopenia due to an increased peripheral destruction from thrombocytopenia resulting from a decreased platelet production. Since the PPR can be reduced in both patients with ITP and megakaryocyte hypoplasia, this parameter does not discriminate well between disorders of production and destruction. In contrast, the MPL appears to be a good parameter to distinguish between thrombocytopenia due to decreased production and increased destruction. The MPL, however, is dependent on several factors, including the platelet count. Apart from a random loss of platelets from the circulation, there exists a con-
stant basal loss of platelets in order to support normal hemostasis and vascular integrity. This fixed number of platelets per day has been estimated to be about 18% of the normal rate of platelet turnover (or a mean of $7.1 \times 10^9$ platelets/L/day). These findings implicate that thrombocytopenia per se may cause a significant reduction in platelet survival, especially when platelet counts are low (below $50 \times 10^9$/L). Therefore, in thrombocytopenic patients accelerated rates of platelet removal due to random mechanisms can be appreciated only if their platelet survival is significantly shorter than is expected based on their platelet count.$^{35,37}$

Platelet kinetic studies in ITP have consistently revealed a significant decrease in platelet life span, but results on the PPR have shown divergent results. Early studies showed a 2-8 times increase in PPR compared to healthy controls.$^{11,38,39}$ Many subsequent publications however, have reported that the PPR is often normal and even reduced in large subgroups of ITP patients.$^{17,18,35,40-47}$ The discrepancies among the studies might be explained by differences in patient characteristics, the use of homologous instead of autologous platelets in the early studies, since a significant reduction in platelet survival times in homologous compared to autologous has been demonstrated. A differential effect of the platelet antibody on the used platelet label ($^{111}$In versus $^{51}$Cr) might also be of influence.$^{17,44}$ Pooled data indicate that about 38% of patients have a decreased PPR, 44% a normal PPR, and only 18% an increased PPR.$^{30}$

Regarding thrombokinetics in MDS only scarce inconsistent data exist which will be summarized in the discussion section of Chapter III.

**Plasma glycocalcin and the glycocalcin-index**

The platelet membrane glycoprotein (GP) Ib (170,000 daltons) is composed of a larger $\alpha$-chain (150,000 daltons) and a smaller $\beta$-chain (23,000 daltons) and is present in high density on the platelet surface (about 25,000 copies per platelet). GPIb is also expressed on megakaryocytes and in limited numbers on endothelial cells. GPIb functions as a receptor for von Willebrand factor and is therefore responsible for platelet adhesion to the subendothelium as the initial event in hemostasis. The $\alpha$-chain has a small hydrophobic transmembrane portion, and a larger carbohydrate-rich hydrophilic external part, called glycocalcin (GC; 135,000 daltons). GC can be cleaved from GPIb$\alpha$ by a variety of proteases, including one or more platelet-derived calcium-dependent proteases. With platelet disintegration such calcium-dependent enzymes are released into the calcium-rich plasma, leading to release of GC in the circulation.$^{48,49}$ GC is soluble and circulates in normal plasma in a concentration of 1-3 µg/ml. In Bernard-Soulier syndrome, a rare autosomal recessive inherited disorder, characterized by giant platelets, mild thrombocytopenia and bleeding tendency, membrane GPIb and
Plasma GC levels are dependent on multiple factors, such as the platelet mass and the platelet turnover. When the platelet survival time is normal and there is absence of platelet sequestration, GC levels reflect the platelet count. Thus, in patients with thrombocytopenia GC concentration is increased, whereas in patients with severe thrombocytopenia due to defective platelet formation as in aplastic anemia or amegakaryocytic thrombocytopenia, plasma GC levels are virtually undetectable. In patients with a normal or increased number of megakaryocytes and thrombocytopenia resulting from increased platelet destruction as in ITP, the expected decrease in GC level based on the reduced platelet count is compensated by an increased release of GC in the circulation due to enhanced platelet disintegration, leading to a net normal or (slightly) increased plasma GC concentration. These findings indicate that the GC level is also dependent on the platelet turnover. To determine whether the GC level is in accordance with the platelet count, the GC-index (GCI) was introduced, in which the GC value is normalized for the individual platelet counts. The GCI was within the normal range in patients with aplastic anemia, amegakaryocytic thrombocytopenia or essential thrombocytopenia, but strongly increased in thrombocytopenic patients with normal or increased megakaryocytes, such as active ITP, thrombotic thrombocytopenic purpura, and drug-induced thrombocytopenia, indicating that in the latter diseases the plasma GC is mainly the result from increased platelet turnover. The GCI is significantly (inversely) associated platelet survival, indicating that the GCI is a parameter of platelet destruction. Several authors have described that the GCI is an aid in discriminating thrombocytopenia due to increased platelet destruction from thrombocytopenia due to impaired production. Others reported that the specificity and sensitivity of GC and the GCI is too low to be of value in distinguishing the cause of thrombocytopenia, because the GCI can also be increased in liver cirrhosis, renal failure, acute and chronic leukemia. In leukemia extremely high GC and high GCI levels have been found, probably because in this disease enzymes such as leukocyte elastase released from destructed myeloid cells may cleave GC from platelets.

**Thrombopoietin**

The hematopoietic growth factor TPO, the ligand for c-Mpl (TPO receptor), is the primary regulator of the megakaryopoiesis. TPO induces megakaryocyte proliferation, differentiation, maturation and endomitosis, and inhibits apoptosis of immature megakaryocytes. The final stage of platelet formation and release is not dependent on TPO, and actually may be inhibited by large amounts of TPO. In TPO or c-Mpl knock-out mice the production of megakaryocytes and platelets is reduced to about 10% of nor-
mal. The production of TPO by especially the liver and the kidney is constitutively and not influenced by the platelet count. Instead, the circulating TPO level is directly regulated by the total number of c-Mpl bearing cells, i.e. the platelet and the megakaryocyte mass, and therefore in thrombocytopenic patients in particular by the megakaryocyte mass. This implicates that when thrombocytopenia is associated with megakaryocyte hypoplasia, TPO level are extremely high, whereas patients with ITP, with a normal or increased megakaryocyte mass, have normal or slightly elevated TPO levels.

In MDS, results on circulating TPO levels have been less uniform. Most studies showed that the endogenous TPO level is elevated in MDS patients, especially in those with RA. This might in part be due to a decreased number of TPO receptors per platelet and megakaryocytes in MDS, although other studies have not confirmed these results. In RA patients, but not in patients with RAEB or RAEB-t, both the platelet and megakaryocyte counts correlate inversely with the endogenous TPO level. These findings indicate that the physiological mechanism for regulating the endogenous TPO level is conserved in RA patients, but deranged in RAEB/RAEB-t. This derangement may be explained by the expression of the TPO receptor on blast cells. In keeping with this notion are results that TPO stimulates the in vitro proliferation of blasts, especially in high-risk MDS.

**AIM OF THE THESIS**

The studies described in this thesis focus on disease mechanisms of MDS and ITP, in particular on megakaryopoiesis and platelet production. As described above these disease mechanisms have not been fully elucidated. In an attempt to clarify some of these mechanisms, the following questions were studied:

1. Is the thrombocytopenia observed in low-risk MDS caused by both a reduced platelet production due to premature megakaryocyte cell death and increased peripheral platelet destruction? (Chapter III)

2. Since caspase-3 independent cell death was observed in megakaryocytes from low-risk MDS patients (Chapter III), what are the potential mechanisms of cell death in erythroid precursors from these patients? (Chapter IV)
3. Can the reduced platelet production in ITP patients be explained by an increased intramedullary destruction of platelets and/or megakaryocytes and is this reflected by the level of the glycocalcin-index? (Chapter V)

4. Do megakaryocytes from ITP patients demonstrate signs of premature cell death and is this reflected by an elevated glycocalcin-index? (Chapter VI)

5. Do parameters, such as PPR and glycocalcin-index and mean platelet life, have clinical implications for the treatment of ITP patients? (Chapter VII)
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