Studies on megakaryopoiesis in patients with myelodysplasia and idiopathic thrombocytopenic purpura
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Megakaryocytic dysfunction in myelodysplastic syndromes and idiopathic thrombocytopenic purpura is in part due to different forms of cell death

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ABSTRACT

Platelet production requires compartmentalized caspase activation within megakaryocytes. This eventually results in platelet release in conjunction with apoptosis of the remaining megakaryocyte. Recent studies have indicated that in low-risk myelodysplastic syndromes (MDS) and idiopathic thrombocytopenic purpura (ITP), premature cell death of megakaryocytes may contribute to thrombocytopenia. Different cell death patterns have been identified in megakaryocytes in these disorders. Growing evidence suggests that, besides apoptosis, necrosis and autophagic cell death, may also be programmed. Therefore, programmed cell death (PCD) can be classified in apoptosis, a caspase-dependent process, apoptosis-like, autophagic and necrosis-like PCD, which are predominantly caspase-independent processes. In MDS, megakaryocytes show features of necrosis-like PCD, whereas ITP megakaryocytes demonstrate predominantly characteristics of apoptosis-like PCD (para-apoptosis). Triggers for these death pathways are largely unknown. In MDS, the interaction of Fas/Fas-ligand might be of importance, whereas in ITP antiplatelet autoantibodies recognizing common antigens on megakaryocytes and platelets might be involved. These findings illustrate that cellular death pathways in megakaryocytes are recruited in both physiological and pathological settings, and that different forms of cell death can occur in the same cell depending on the stimulus and the cellular context. Elucidation of the underlying mechanisms might lead to novel therapeutic interventions.
INTRODUCTION

Megakaryocytes arise from pluripotent hematopoietic stem cells that undergo lineage commitment, proliferation and differentiation under the influence of cytokines, in particular thrombopoietin (TPO). As a result of endomitosis, megakaryocytes become polyploid cells with a large cytoplasmic mass. This enables each megakaryocyte to produce 1000–3000 platelets. The exact mechanism of platelet production remains unclear. Current evidence supports the mechanism initially suggested by Wright in 1906, that platelets bud off from cytoplasmic pseudopodial extensions (proplatelets). These proplatelets are created from an abundant cytoplasmic reservoir of membranes (demarcation membrane system).\(^1\) After the release of platelets, the remaining senescent megakaryocyte, consisting of a nucleus and a thin margin of cytoplasm (denuded megakaryocyte), undergoes apoptosis, a common form of programmed cell death (PCD).\(^2,3\) However, the apoptotic program is also required for proplatelet formation and the release of mature platelets. Activation of caspase-3 and -9 has been demonstrated not only in senescent megakaryocytes but also in maturing and proplatelet-bearing megakaryocytes.\(^4\) In contrast to senescent megakaryocytes, no DNA fragmentation could be detected in maturing megakaryocytes, suggesting that caspase activation is compartmentalized. Consistent with these findings, the cytoplasmic distribution of activated caspase-3 showed a granular pattern in maturing megakaryocytes, whereas in senescent megakaryocytes a diffuse cytoplasmic staining pattern was observed. Thus, after proplatelet formation caspase activation switches from a circumscript to a diffuse form, thereby inducing apoptosis in senescent megakaryocytes. This notion is supported by observations of diminished proplatelet formation by caspase inhibitors and overexpression of antiapoptotic protein Bcl-2.\(^4\) 

Thus, in normal physiology platelet production and megakaryocyte apoptosis are closely related events. In diseases, however, premature PCD might disrupt platelet formation. This has been demonstrated in myelodysplastic syndromes (MDS) and idiopathic thrombocytopenic purpura (ITP). This review discusses the role of PCD in these disorders and summarizes some recent developments concerning PCD.
PROGRAMMED CELL DEATH

PCD is defined as an active, controlled, process of sequential, in principle reversible, events leading to cell death. PCD requires the activity of specific genes, is not associated with an inflammatory response and plays a major role in both normal development and disease. These characteristics distinguish PCD from accidental necrosis. Apoptosis is often used as a synonym of PCD. However, certain forms of necrosis and autophagic cell death are also programmed events. Therefore, PCD can be subdivided into apoptosis (type I), autophagic cell death (type II) and necrosis-like PCD (type III). This classification is primarily based on morphology. However, many other techniques to assess cell death exist. Most of these methods are not sensitive and specific enough to diagnose cell death independently. Therefore, it is advised to use multiple techniques simultaneously. A summary of frequently used techniques is given in Table 1.

Apoptosis (type I PCD)
Apoptosis is morphologically characterized by chromatin condensation, nuclear fragmentation, cell surface blebbing, cell shrinkage and the formation of apoptotic bodies. The induction of classic apoptosis involves the sequential activation of initiator and effector caspases, which are proteolytic enzymes that degrade essential cellular targets. Caspase-3, the main effector caspase, can be activated by an extrinsic pathway involving cellular death receptors, and an intrinsic (mitochondrial) pathway involving cytochrome c, Apaf-1 and caspase 9. The mitochondrial pathway can also induce caspase-independent apoptosis (also called apoptosis-like PCD) (Figure 1).

Autophagic cell death (type II PCD)
Type II PCD can develop from autophagy (self eating), characterized by sequestration of cellular organelles in double-membraned vacuoles (autophagosomes), which fuse with lysosomes leading to degradation of their content. However, frequently autophagy, at least initially, functions as a protective mechanism for cell destruction. In situations of starvation, the cells maintains ATP production by reducing the amount of cytoplasm and organelles into autophagosomes. Autophagic cell death has predominantly been observed when the apoptotic pathway is blocked, suggesting that cells die preferentially by apoptosis and that apoptosis is a faster process than autophagic cell death.
Table 1. A selection of methods of cell death detection applicable to tissue sections.\textsuperscript{9,10}

<table>
<thead>
<tr>
<th>Methods</th>
<th>Type of cell death</th>
<th>Stage of cell death</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>Apoptosis</td>
<td>Late</td>
<td>Gold standard, but morphology alone is insensitive</td>
</tr>
<tr>
<td></td>
<td>Autophagic cell death</td>
<td></td>
<td>Gold standard (two-membraned vacuoles)</td>
</tr>
<tr>
<td></td>
<td>Necrosis</td>
<td>Late</td>
<td>Gold standard, not distinguishable from necrosis-like PCD</td>
</tr>
<tr>
<td><strong>Histochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUNEL/ ISEL</td>
<td>Apoptosis</td>
<td>Intermediate and late</td>
<td>Pretreatment that deteriorates DNA can reduce sensitivity \newline False-positivity may occur in: necrosis, cells in process of DNA repair, pretreatment that induces DNA breaks \newline Helpful in diagnosing apoptosis when used in combination with morphology \newline Applicable to archival material</td>
</tr>
<tr>
<td>Caspases</td>
<td>Apoptosis</td>
<td>Intermediate and late</td>
<td>Helpful in diagnosing apoptosis when used in combination with morphology \newline Correlates well with morphology and TUNEL \newline Applicable to archival material</td>
</tr>
<tr>
<td>LC-3</td>
<td>Autophagic cell death</td>
<td>Autophagosome</td>
<td>Correlates well with morphology of autophagosomes</td>
</tr>
</tbody>
</table>

*Abbreviations:* LC-3, light chain-3; ISEL, in situ end labeling; TUNEL, TdT-mediated dUTP-biotin nick end labeling
Figure 1. Schematic model of pathways leading to cell death. Predominantly mitochondrial pathways are depicted. Classical apoptosis can be triggered by the extrinsic (A) and intrinsic (mitochondrial) pathway (B). Both pathways can be triggered by death receptor-mediated caspase-8 activity. Numerous other stress responses converge on the mitochondria and can promote the onset of MPT. MPT can subsequently lead to MOMP. Depending on multiple factors, such as the speed of MPT, the number of mitochondria involved and the availability of adenosine triphosphate (ATP), either apoptosis, necrosis or autophagy occurs. The ovals represent a refinement of the traditional classification of cell death.7 **Abbreviations:** AIF, apoptosis inducing factor; CD, cell death; EndoG, endonuclease G; MPT, mitochondrial permeability transition; MOMP, mitochondrial outer membrane permeabilization; ROS, reactive oxygen species.

Necrosis-like PCD (type III cell death)
Necrosis is an unregulated process, characterized by rapid cell and organelle swelling, loss of plasma membrane integrity, ATP depletion, ion deregulation and activation of degradative enzymes. However, regulated forms of necrotic cell death (called necrosis-like PCD or programmed necrosis) have also been observed, especially under conditions (i.e. viral infections) in which apoptosis is inhibited.5–8
Cross-talk between types of cell death and mitochondria
Between the different types of cell death, flexibility exists making it possible that apoptosis can switch to necrosis and autophagy depending on the cellular context. Several studies have shown that the inhibition of the apoptotic machinery can trigger a switch from apoptosis to necrosis\textsuperscript{6,8} or autophagy.\textsuperscript{11} In these processes, mitochondrial dysfunction plays an important role.\textsuperscript{7} Mitochondria can integrate cell death signals and represent a nexus at which different pathways interact.\textsuperscript{12} Crucial events at the mitochondrial level are: opening of mitochondrial permeability transition (MPT) pores in the inner mitochondrial membrane, loss of the mitochondrial transmembrane potential and mitochondrial outer membrane permeabilization (MOMP)\textsuperscript{7,8,13} (Figure 1). The intensity of the stimulus leading to MPT and the cellular context determines which type of cell death develops.\textsuperscript{7,8}

PROGRAMMED CELL DEATH IN MDS

Apoptosis in MDS: evidence and controversies
MDS are a heterogeneous group of clonal hematopoietic stem cell disorders, characterized by a dysplastic and ineffective hematopoiesis. The alterations in the hematopoietic cells are thought to result from irreversible DNA damage within a hematopoietic stem cell and an accumulation of multiple genetic lesions during hematopoiesis. The precise pathogenesis of MDS is not elucidated, but numerous studies (reviewed by Parker and Mufti;\textsuperscript{14} Liesveld et al.;\textsuperscript{15} Yoshida and Mufti\textsuperscript{16}) indicate that enhanced intramedullary apoptosis may be an important disease mechanism, especially in explaining the paradox between bone marrow hypercellularity and peripheral cytopenias, in particular in low-risk MDS. However, studies on apoptosis have also been conflicting and disagreement regarding the degree and extent of apoptosis, the involvement of stromal cells and the clinical implications of apoptosis in MDS bone marrow, remains. The evidence and controversies regarding apoptosis in MDS have been reviewed extensively elsewhere.\textsuperscript{15–17}

Thrombocytopenia in low-risk MDS and PCD of MDS megakaryocytes
Thrombocytopenia occurs in 30–50\% of patients with MDS and may result in serious bleeding complications. Isolated thrombocytopenia is the presenting manifestation in 5–10\% of MDS patients, and may then be mistaken for ITP.\textsuperscript{18}
Increased platelet destruction?
Up to 50% of patients have a decreased platelet lifespan, suggesting that increased peripheral platelet destruction mediated by (non-)immune mechanisms might contribute to thrombocytopenia in MDS. Findings that several immunomodulatory agents and splenectomy lead to an increase in platelet counts in some MDS patients may support this notion.

Decreased platelet production.
However, thrombocytopenia in MDS is mainly caused by ineffective platelet production resulting from impaired proliferation and differentiation of megakaryocytes and their precursors. Morphological studies of MDS bone marrow have shown increased numbers of abnormal megakaryocytes, in particular micromegakaryocytes with a low peak ploidy number (4–8N), suggesting an expansion of megakaryocytic precursors, an arrest in terminal megakaryocyte differentiation and impaired nuclear development. Other dysplastic features include large mononuclear forms, multiple separate nuclei, dissociation between cytoplasmic and nuclear maturation and megakaryocytic hypogranulation. Although not all megakaryocytes in MDS appear abnormal on light microscopy, cytogenetic studies show that the majority of megakaryocytes are involved in the MDS clone, even when micromegakaryocytes are not analyzed.

Several studies have shown a defective in vitro megakaryopoiesis in MDS. In many MDS cases, megakaryocyte progenitor growth was unresponsive to recombinant TPO. This defective response is probably due to deregulated TPO receptor-mediated signaling pathways, as a lack of serum TPO, a decreased expression of c-Mpl (TPO-receptor) or mutations in the c-Mpl gene have been excluded.

PCD in MDS megakaryocytes.
Studies on apoptosis in MDS megakaryocytes are scarce compared to erythroid and myeloid precursors. This is in part due to the low number of megakaryocytes and their vulnerability during bone marrow sample processing. Elevated numbers of denuded megakaryocytes in bone marrow biopsies from MDS patients have been reported and have been ascribed to apoptosis on the basis of earlier reports concerning the ultrastructure of senescent murine megakaryocytes. The observations in MDS, however, were solely based on light microscopy. Several studies using electron microscopy, TdT-mediated dUTP-biotin nick end labeling (TUNEL) or in situ end labeling (ISEL) have reported apoptosis in megakaryocytes. Other authors, however, reported that they could not identify apoptosis in megakaryocytes, perhaps as a result of bone marrow sample preparation techniques or owing to a lineage-restricted propen-
sity for apoptosis. Similarly, a recent study\textsuperscript{34} using ISEL demonstrated apoptosis in only 4\% of megakaryocytes, which were predominantly micromegakaryocytes. These findings are largely consistent with results from our own study,\textsuperscript{19} in which MDS megakaryocytes were negative for activated caspase-3 and showed no ultrastructural features of apoptosis. The megakaryocytes demonstrated ultrastructural changes resembling necrosis-like cell death, including clumping and random degradation of nuclear chromatin and cytoplasmic vacuoles (Figure 2c and Table 2). How these megakaryocytic changes arise, is not clear. Preliminary results suggest a role for the Fas/Fas-ligand (FasL) system. Immunohistochemical staining of MDS megakaryocytes showing necrosis-like morphology was positive for Fas and FasL (Blom et al., unpublished observations, 2005). Increased death signals, in particular the Fas/FasL system might play an important role in inducing intramedullary apoptosis in MDS.\textsuperscript{35} Apart from inducing apoptosis, stimulation of Fas and other death receptors (i.e. tumor necrosis factor-receptor 1) can also trigger caspase-independent pathways leading to necrosis-like PCD.\textsuperscript{36} Crucial in Fas-mediated signaling to either apoptotic or necrosis-like PCD is the Fas-associated death domain (FADD). When caspases are inhibited, signaling via FADD leads to necrosis-like PCD. Alternatively, FADD-induced necrotic PCD can be reverted to apoptosis by degradation of receptor-interacting protein 1.\textsuperscript{36} Thus, as it is the cellular context that determines whether stimulation of Fas triggers apoptotic or necrosis-like PCD, increased Fas/FasL expression in bone marrow cells of MDS patients might also be related to the presence of necrosis-like PCD. A similar switch in PCD might occur dependent on in vivo or in vitro conditions. Caspase-independent cell death might occur primarily in bone marrow megakaryocytes, but may switch to apoptosis when the cells are taken from their microenvironment. Although some in vitro studies suggest that MDS stromal cells induce apoptosis in hematopoietic cells,\textsuperscript{14} in vivo it may be possible that prosurvival signals provided by the microenvironment inhibit the apoptotic pathway. These signals might, in concert with the intrinsic cellular defects in signaling pathways,\textsuperscript{37} make the MDS megakaryocytes vulnerable for necrosis-like PCD. When the remaining prosurvival signals disappear owing to the detachment of megakaryocytes from the microenvironment, the cells might become programmed to the apoptotic route.

In summary, the mechanisms underlying defective megakaryopoiesis in MDS are not completely elucidated. PCD of mature megakaryocytes probably contributes to thrombocytopenia; however, apoptosis may play a secondary role.
Is platelet production impaired in ITP?
In classical ITP, platelet lifespan is greatly reduced owing to accelerated immune-mediated peripheral platelet destruction, predominantly in the spleen. As a result, platelet production is considered to be compensatorily increased, reflected by an elevated number of megakaryocytes in the bone marrow and by an increased platelet turnover determined with radiolabeled platelet studies. However, many observations have questioned this traditional view of ITP. Early morphological studies revealed that the number of megakaryocytes in ITP patients is often normal instead of increased, and
<table>
<thead>
<tr>
<th>Ultrastructure</th>
<th>ITP</th>
<th>MDS</th>
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<tbody>
<tr>
<td>Nuclear changes</td>
<td>Nucleus in periphery of cell Chromatin condensation: Some MKs: crescent-shaped; Majority of MKs: without margination (compatible with para-apoptosis)</td>
<td>Nucleus centrally in the MK Noncondensed chromatin, partly lying in spherical speckles/ small clumps; no nucleoli; smooth nonlobulated outlines</td>
</tr>
<tr>
<td>Cytoplasmic changes</td>
<td>Vacuoles, swelling of endoplasmic reticulum, mitochondria with disrupted cristae and distended DMS; enlarged peripheral zone (para-apoptosis)</td>
<td>Cytoplasmic vacuoles; mitochondrial disruption reduced numbers of granules, disordered DMS</td>
</tr>
</tbody>
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| DNA fragmentation | | |
|------------------|------------------|
| TUNEL/ ISEL | Normal in children with acute ITP and chronic ITP (bone marrow aspirates) | Conflicting results (see text) |

<table>
<thead>
<tr>
<th>Caspase activity</th>
<th>ITP</th>
<th>MDS</th>
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</thead>
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<tr>
<td>Immunohistochemistry</td>
<td>Megakaryocytes with apoptotic morphology were caspase-3 positive</td>
<td>Caspase-3: conflicting results: negative /positive</td>
</tr>
<tr>
<td>Possible stimuli</td>
<td>Factors in ITP plasma induce abnormalities</td>
<td>Fas/Fas-ligand?</td>
</tr>
<tr>
<td>Possible inhibitors</td>
<td>Prednisolone, IVIG</td>
<td>unknown</td>
</tr>
</tbody>
</table>

**Abbreviations:** DMS, demarcation membrane system; ISEL, in situ end labeling; ITP, idiopathic thrombocytopenic purpura; IVIG, intravenous immunoglobulins; MDS, myelodysplastic syndromes; MK, megakaryocyte; PCD, programmed cell death; TUNEL, TdT-mediated dUTP-biotin nick end labeling; ^a Blom et al, unpublished results
that an increased number of megakaryocytes does not always imply increased platelet production. Most megakaryocytes in these observations were morphologically altered and surrounded by a greatly diminished number of platelets. In addition, platelet kinetic studies have identified large subgroups of ITP patients with a decreased or normal platelet turnover.

**Morphological alterations of megakaryocytes**

Morphological changes in ITP megakaryocytes, such as extensive cytoplasmic vacuolization, hypogranularity and smoothing of the cell membrane, were already described by Frank in 1915, and later confirmed by others. It was argued that these alterations were artifacts induced by fixation and/or staining methods. Similar abnormalities, however, were found using phase-contrast microscopy, by which cells can be examined in the living and unstained state. Ultrastructurally, a majority of ITP megakaryocytes show alterations, including cytoplasmic vacuoles owing to swelling of mitochondria and endoplasmic reticulum and chromatin condensation. This morphology resembles features of PCD, including apoptosis (confirmed by detection of caspase-3 activity) and para-apoptosis, a form of active caspase-3-negative, TUNEL- and ISEL-negative apoptosis-like PCD (Figure 2a–b and Table 2). Para-apoptotic megakaryocytes have also been described in idiopathic myelofibrosis and GATA-1low mice. In these mice, blocked megakaryocyte maturation results in an accumulation of defective megakaryocytes showing increased neutrophil emperipolesis. Subsequent release of neutrophilic proteases in the megakaryocyte cytoplasm might induce para-apoptosis.

An alternative explanation for the extensive cytoplasmic vacuoles in ITP megakaryocytes might be autophagy. Considering a state of compensatorily increased megakaryopoiesis and therefore a state of increased metabolic demand and relative nutrient deficiency, autophagy might be a mechanism for generating enough energy to maintain cell metabolism. Alternatively, autophagy might be a way of sequestering and degrading specific pathogens, such as immunoglobulins in the case of ITP. In this situation, autophagy may end in type II PCD or perhaps apoptosis, when autophagy has reached its limits. The observed cytoplasmic vacuoles in megakaryocytes of ITP patients, however, appear of non-lysosomal origin. They are mainly not double-membraned, mostly empty and appear to originate predominantly from dilated organelles.

**The etiology of the megakaryocyte alterations in ITP**

Some have ascribed these megakaryocytic changes to the compensatorily increased megakaryopoiesis, as similar abnormalities have been described in megakaryocytes from animals made thrombocytopenic by thrombocytopenia. Increased thrombocy-
topenia-induced thrombopoiesis should theoretically lead to elevated numbers of de-nuded megakaryocytes.\textsuperscript{3} To our knowledge, this has not been reported in ITP bone marrow.\textsuperscript{47,48} Furthermore, the damaged megakaryocytes found in ITP\textsuperscript{43} show no ultrastructural resemblance to these denuded megakaryocytes.

There is evidence that factors in ITP plasma, possibly antiplatelet autoantibodies, are responsible for the megakaryocyte alterations in ITP. Morphological alterations resembling those found in ITP megakaryocytes could be induced in megakaryocytes from healthy persons within 2 h after intravenous injections of plasma from ITP patients.\textsuperscript{42} We found corresponding results with electron microscopy in megakaryocytes cultivated in the presence of ITP plasma.\textsuperscript{43} It has been shown that antiplatelet autoantibodies can recognize antigens on megakaryocytes and can suppress the in vitro production and maturation of megakaryocytes,\textsuperscript{49} suggesting that platelet production in many ITP patient is suppressed.

CONCLUDING REMARKS

In the last decade, the knowledge on PCD has expanded substantially. The classical division of cell death in apoptosis, which is often used as a synonym of PCD, and necrosis has evolved to a broad spectrum of PCD in which both apoptosis, necrosis-like PCD, autophagic cell death and mixed forms have their place. PCD is an essential element of normal and pathological cell physiology. This also accounts for the megakaryopoiesis. Like many cells, megakaryocytes possess several pathways that lead to cell death. In normal physiological conditions, elements of the apoptotic system are required for the formation of platelets; in diseases, such as MDS and ITP, inappropriate, apoptotic and non-apoptotic, PCD of megakaryocytes occurs. In ITP, megakaryocytes are intrinsically normal cells and PCD is induced by external factors. MDS megakaryocytes undergo increased PCD probably as a consequence of extensive intrinsic defects and a perturbed response to (possible aberrant) extrinsic factors from the bone marrow microenvironment. Future research has to focus on the exact triggers, mechanisms and clinical implications of PCD in these disorders, in order to generate more insight into the pathophysiology of thrombocytopenia in ITP and MDS and to develop new treatment strategies, especially for patients with refractory disease and symptomatic thrombocytopenia. Although blocking caspases often stops the apoptotic process, PCD is frequently not prevented and other, caspase-independent, forms of cell death.
develop instead. As mitochondrial dysfunction (especially MOMP) represents the ‘point of no return’ of cell death, preventing mitochondrial dysfunction or common regulatory mechanisms upstream of mitochondrial dysfunction might be promising for novel therapeutic interventions. Although a major problem in developing these strategies is drug specificity, so that interventions do not influence normal cell physiology, numerous strategies of inhibition of cell death that target death receptors and their ligands, p53, stress kinases and proteases, and MOMP, are currently investigated and in some instances already available for clinical use.\(^{13}\)

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