Immune thrombocytopenia (ITP) is a complex disease. The pathogenic and clinical heterogeneity of ITP is reflected by reports on variability in patient history and treatment response, in concert with recent evidence from mechanistic studies. Programmed cell death (PCD) pathways are thought to play a peculiar role in the megakaryocyte lineage in terms of hemostasis and the generation and function of megakaryocytes and platelets; unbalanced genetic or environmental disturbances of these tightly regulated pathways may cause thrombocytopenia. Dysregulated PCD has also been linked to peripheral platelet destruction, intramedullary apoptosis, and inefficient thrombopoiesis in ITP. In this article, we discuss novel and controversial findings on the role of PCD in the megakaryocyte lineage and their potential implications in terms of pathogenesis, diagnosis, and treatment of ITP.
the cytosol. Once released, cytochrome c assembles with apoptotic protease-activating factor 1 (APAF1) and the initiator caspase, procaspase-9, to form the apoptosome, which activates caspase-9 and downstream effector caspases. The activity of Bak and Bax is tightly controlled by anti-apoptotic Bcl-2 family members, of which Bcl-2, Bcl-xL, and Mcl-1 are expressed in megakaryocytes. Bcl-xL and Mcl-1 have been shown to coordinately regulate megakaryocyte survival, whereas Mcl-1, but not Bcl-xL, seems dispensable for platelet survival. A number of factors have been shown to trigger the intrinsic pathway to apoptosis in platelets, including chemical stimuli, biomechanical rheological forces, and temperature. The so-called extrinsic pathway to apoptosis is triggered by ligation of “death receptors” belonging to the tumor necrosis factor (TNF) receptor superfamily that contain an intracellular death domain, which can recruit and activate the initiator caspase, procaspase-8. This leads to direct activation of downstream effector caspases by caspase-8, such as caspase-3, -6, or -7. The extrinsic pathway can hence bypass the Bcl-2 family and mitochondria. However, in some cells (eg, hepatocytes, neutrophils) caspase-8—mediated cleavage of the pro-apoptotic BH3-only protein Bid to its truncated form, tBid, has been described, which leads to Bak- and Bax-dependent MOMP. Although current evidence suggests that megakaryocytes possess both intrinsic and extrinsic pathways to apoptosis, molecular and functional aspects of the extrinsic pathway of apoptosis remain to be explored in the megakaryocyte lineage.

**PROCESS OF PLATELET PRODUCTION: A ROLE FOR CASPASE-DEPENDENT PATHWAYS?**

The process of platelet release involves the formation of megakaryocyte cytoplasmic extensions called proplatelets and intracellular membranes, so-called demarcation membranes, which are probably internal invaginations of the cytoplasmic membrane, that eventually may serve as a membrane reservoir for the generation of proplatelets. While proplatelets will engender functional platelets that are released in the circulation, the remaining senescent megakaryocyte body will remain in the bone marrow as so-called denuded megakaryocyte, then undergo apoptosis, and ultimately be phagocytosed by macrophages. Since the observation that platelet release from megakaryocytes coincides with the onset of apoptotic morphology, a significant body of literature has been accumulated to support the view that the apoptotic machinery is involved in the complex cytoplasm reorganization events required for platelet formation and shedding. De Botton et al proposed that platelet formation involves compartmentalized and caspase-dependent apoptosis.

In another study, ligation of the death receptor Fas (also called CD95) by either agonistic anti-Fas antibody or soluble Fas resulted in a significant increase in proplatelet extensions and production of functional platelets. In both studies, the pan-caspase inhibitor zVAD-fmk was employed to prove caspase-dependence. Mice constitutively overexpressing pro-survival Bcl-2 in hematopoietic cells, or lacking the proapoptotic BH3-only protein Bim, exhibit mild thrombocytopenia. Interestingly, thrombocytopenic individuals with a mutation variant of cytochrome c, ie, substitution of glycine by serine at residue 41 (G41S), which exhibits normal redox function but enhanced pro-apoptotic activity, present with defective platelet formation. Electron microscopy of bone marrow from these patients showed intramedullary naked megakaryocyte nuclei and platelets, indicative of dysregulated megakaryopoiesis with premature release of platelets into the marrow space rather than into sinusoïds.

The concept that apoptosis is required for platelet generation has been challenged by recent genetic deletion or pharmacologic inhibition studies, which showed that caspase-dependent pathways are dispensable for platelet production, but instead might lead to accelerated megakaryocyte death. In 2007, Mason et al reported that loss of proapoptotic Bak and Bax prolonged platelet lifespan, but had no impact on platelet production. In support of these data, Kozuma et al showed that megakaryocytes of transgenic mice overexpressing the prosurvival protein Bcl-2 exhibit normal ability for in vitro platelet production; interestingly, early megakaryocyte differentiation was impaired. Furthermore, mice with megakaryocyte-specific loss of caspase-9 exhibited normal platelet counts and functional platelets, as well as normal serum levels of thrombopoietin (TPO), the major cytokine regulator of megakaryopoiesis. Instead, caspase-9 was indispensable for apoptotic death of megakaryocytes and platelets. Similarly, deletion of the prosurvival protein Bcl-xL was shown to trigger megakaryocyte apoptosis associated with failure of platelet production, a defect that could be rescued by loss of Bak and Bax. Together, these data suggest that the intrinsic pathway to apoptosis in megakaryocytes is not required for platelet production, but rather initiates PCD.

The role of caspase-dependent pathways in the generation of platelets by megakaryocytes remains to be further explored. For instance, the possibility exists that certain non-apoptotic functions of caspases are required for platelet generation. Other proposed explanations for the conflicting data relate to experimental factors including the toxicity of the caspase inhibitor zVAD-fmk in megakaryocytes, off-target effects of overexpressed proteins or pharmacological inhibitors (such as zVAD-fmk), and properties of in vitro culture systems or cell lines.
In view of the reported data, even if conflicting, it is conceivable that aberrant blockade or activation of apoptotic pathways in megakaryocytes may lead to thrombocytopenia. Better insights into the molecular mechanisms that regulate platelet generation are therefore required and might provide novel targets for drug treatment in ITP or lead to the identification of patient subgroups with a genetic defect of thrombopoiesis, such as indicated by the example of the cytochrome G41S mutation. Future studies are urgently required to dissect the exact biochemical pathways and enzymatic processes that contribute to megakaryocyte development and function at different stages of megakaryopoiesis and platelet shedding, including non-apoptotic or caspase-dependent pathways, and even alternative cell death pathways, such as autophagy and programmed necrosis.

MEGAKARYOCYTE DEATH IN ITP

Morphological alterations of ITP megakaryocytes in the bone marrow have been described by several investigators and even before the infusion experiment by Dr William Harrington in 1950. In a recent ultrastructural study by Houwerzijl et al, ITP megakaryocytes were reported to exhibit cytoplasmic vacuoles and ultrastructural features of apoptosis and para-apoptosis. The percentage of damaged megakaryocytes increased with advanced stages of differentiation, and reached about 80% of mature megakaryocytes in this study. Significantly fewer immature megakaryocytes showed morphologic alterations. The damaged cells were often surrounded by macrophages and neutrophils, presumably stimulating a phagocytic response and an inflammatory reaction, that is eventually evoked by the non-apoptotic form of cell death. Exposure of megakaryocytes from healthy individuals to ITP plasma in vitro, or after transfusion in vivo, has been shown to induce morphological alterations compatible with (para-)apoptosis. It is possible that premature elimination of maturing megakaryocytes, before the initiation of platelet shedding, might lead to ineffective thrombopoiesis rather than to an absolute loss of total megakaryocyte mass. Such a scenario would be compatible with the often normal or even increased megakaryocyte numbers in the bone marrow and the often unaltered plasma TPO levels in ITP patients.

PLATELET PHOSPHATIDYLSERINE EXTERNALIZATION: A ROLE IN ITP?

Externalization of phosphatidylserine (PS) on the surface of cells undergoing apoptosis represents the most universal and best characterized “eat me” signal that leads to recognition as “unwanted self” and clearance of the apoptotic cell by phagocytes. The translocation of PS from the inner to the outer leaflet of the lipid bilayer occurs early in the apoptotic process and involves a nonspecific bidirectional phospholipid flip-flop along with the blockade of an aminophospholipid translocase that normally confines and reverts PS to the cytosolic side of the cell membrane. Interestingly, PS exposure also occurs on the cell surface of platelets upon activation for the platelet to become procoagulant, and to propagate the coagulation process by facilitating the assembly of coagulation factors and activation of tenase and prothrombinase complexes. The level of PS externalization during activation seems to depend on the stimulus as a function of the formation of a mitochondrial permeability transition pore (MPTP) or other induced molecular pathways. Two distinct pathways appear to regulate platelet PS exposure: one calcium-dependent and caspase-independent, and another Bak/Bax-caspase-mediated pathway. Besides its procoagulant function, PS exposure on activated platelets might serve as a regulatory mechanism to avoid potentially deleterious hyperactivity, and, for example, initiate the systemic clearance of circulating activated PS-positive cells by the reticuloendothelial system.

Abnormally increased PS exposure on patient platelets has been reported for adult patients with chronic ITP and for pediatric patients with acute ITP. Winkler et al reported that the frequency of platelets exposing PS was significantly higher in children with acute ITP, and a significantly higher proportion of ITP platelets contained activated caspases-3, -9, and -8 compared to controls. Interestingly, the reported cleavage of caspase-8 suggests activation of the extrinsic, in conjunction with the intrinsic, pathway of apoptosis. In the chronic ITP study, both fresh and aged platelets from patients exhibited significantly higher surface PS levels compared to healthy donor platelets, as assessed by flow cytometric annexin V-staining. In contrast to the study by Winkler et al, exposure of PS appeared to be caspase-independent, but no signs of activation (CD62P, CD154, PAC-1 expression) were found, suggesting that other mechanisms for PS externalization might exist in ITP.

Enhanced PS externalization on platelet surfaces in ITP might lead to exceeding systemic clearance of these cells by the reticuloendothelial system and cause, or aggravate prevailing thrombocytopenia. Future studies are required to support this concept and to explore mechanisms of increased PS externalization, which might involve genetic differences in molecular pathways that determine platelet
autoantibodies also bind to megakaryocytes.41–43 In PRO-APOPTOTIC HUMORAL FACTORS IN ITP senescence,36 apoptosis, activation, responses to hits inflicted by the circulatory environment,37 or susceptibility to detriment caused by autoantibodies or infectious particles.

PRO-APOPTOTIC HUMORAL FACTORS IN ITP

The capacity of platelet surface molecules to trigger platelet apoptosis has been reported for glycoprotein (GP)IIb-IIIa and GPIb-α.5 GPIIbIIIa and GPIb-α represent well known targets of platelet-specific autoantibodies in ITP.5 Injection of rat anti-mouse monoclonal antibody MWR30, targeting the GPIIib subunit of GPIIb-IIa, induced apoptosis in mouse platelets in vivo and was associated with marked thrombocytopenia.38 The injection of anti-GPIIib resulted in platelet PS exposure, caspase-3 activation and mitochondrial inner transmembrane potential depolarization.38 In another study, injection of rabbit polyclonal anti-platelet antibody induced thrombocytopenia that was associated with caspase-3 cleavage in platelets and could be prevented by injection of the caspase inhibitor zVAD-fmk.39 Given this data, it is conceivable that proapoptotic platelet-specific autoantibodies contribute to the abnormally increased PS exposure on patient platelets observed in acute and chronic ITP.34,35

It has been known for many decades that GPIIb-IIIa and GPIb-IX are expressed on the surfaces of megakaryocytes,40 and that anti-platelet and ITP autoantibodies also bind to megakaryocytes.41–43 In addition to the initiation of peripheral platelet destruction, several studies reported the capacity of anti-GPIIb-IIIa and anti-GPIb antibodies in ITP plasma to inhibit megakaryocyte production or maturation, at least in a subpopulation of ITP patients.44–46 In addition to genetic properties, the susceptibility of a megakaryocyte for such suppression by these antibodies might depend on its maturation stage.45

Besides platelet-specific autoantibodies, other humoral factors in ITP plasma regulating lifespan of megakaryocytes and platelets, megakaryopoiesis, or platelet production, might be implicated in disease pathogenesis. For instance, elevated levels of proapoptotic soluble Fas ligand were found in a subgroup of adult patients with chronic ITP.47 Another study reported aberrant levels of soluble tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Obviously, these effector molecules are less specific than autoantibodies; however, given the special role of apoptotic and PCD processes in megakaryocytes and platelets,9 they might participate in certain disease processes, depending on factors such as genetic characteristics, differentiation stage and vulnerability of the target cell.

CELL DEATH REGULATION BY INTRAVENOUS IMMUNOGLOBULIN

Since the seminal observation reported by Paul Imbach et al that intravenous immunoglobulin (IVIG) treatment rapidly elevated platelet counts in ITP,48 its therapeutic effect has been greatly appreciated, and in current guidelines IVIG is still considered a first-line therapy option for this indication.49,50 A number of different mechanisms have been proposed that might eventually act in a mutually non-exclusive manner, including blocking platelet-phagocytosis in the reticuloendothelial system, FcγRIIB-mediated effects, and neutralization by anti-idiotypic antibodies.51,52 Several studies also suggest a potential role of IVIG in the regulation of cell death of platelets and megakaryocytes. Winkler et al reported that the pathologically increased percentage of apoptotic platelets of children with acute ITP is significantly reduced after IVIG treatment.35 Interestingly, IVIG not only inhibited intrinsic, but also extrinsic, death receptor-dependent, pathways of apoptosis, as revealed by cleavage of caspase-8. An inhibitory effect of IVIG on platelet apoptosis has also been shown by Leytin et al in an animal model of ITP.38 Houwerzijl et al reported that IVIG treatment significantly reduced the number of para-apoptotic and apoptotic megakaryocytes and increased the frequency of mature megakaryocytes from 20% to 43%.26

IVIG preparations are derived from pooled plasma of thousands of healthy donors and hence contain the repertoire of the donor population. Studies on IVIG have revealed that healthy individual produce autoantibodies with the capacity to balance apoptotic processes of hematopoietic cells.53,54 Such autoantibodies include agonistic antibodies to Fas,55–57 Siglec-8,58 and Siglec-9,59 blocking antibodies to Fas,55,60 and even anti-idiotypic antibodies to Fas and Siglec-9.61 Whether quantitative or qualitative dysregulation of these autoantibodies is implicated in the pathogenesis in ITP remains to be explored. However, therapeutic effects of IVIG in toxic epidermal necrolysis (TEN) have been linked to the presence of blocking anti-Fas antibodies in IVIG.69 This study suggests that infusion of anti-apoptotic antibodies may restore cellular homeostasis in diseases associated with deregulated apoptotic processes, including ITP.

CONCLUSIONS

ITP is a diagnosis of exclusion. The variability in natural history and response to therapy suggests that ITP comprises heterogeneous disorders,62,63 which might hence be regarded a syndrome.62 This notion is supported by recent insights into the pathogenesis
of ITP, derived from a broad range of experimental studies focusing on different aspects of antibody- and cell-mediated autoimmune responses. Here, we discussed recent, and in part conflicting, evidence, on the role of cell death pathways in a variety of processes that might be implicated in the pathogenesis of ITP (Figure 1). Genetic or acquired defects of these pathways, or altered susceptibility to antibody- or cell-mediated apoptosis, might cause or support thrombocytopenia in ITP, in processes that include intramedullary apoptosis, ineffective thrombopoiesis, and peripheral platelet destruction. A better understanding of these mechanisms might lead to novel therapeutic approaches and to a better patient selection for the treatment of ITP.

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