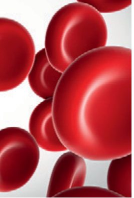

Blood Spotlight

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Apoptosis in megakaryocytes and platelets: the life and death of a lineage

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Despite their profoundly different cellular composition, size, and function, megakaryocytes and platelets both depend on restraint of the intrinsic (or "mitochondrial") apoptosis pathway by BCL-2 family prosurvival proteins for their development and viability. Activation of the pathway contributes to the clearance of megakaryocytes following platelet shedding and constrains platelet lifespan in the circulation. Important questions remain as to how apoptosis is initiated in these cells at steady state and in response to pathophysiological insults. (*Blood*. 2018;131(6):605-610)

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Apoptosis: a specific form of programmed cell death

Originally coined by Kerr and colleagues in 1972 to describe a morphologically distinct mechanism of "cell deletion,"[1] apoptosis is now very clearly defined at the biochemical level. It refers to 2 convergent pathways of programmed cell death: the extrinsic and the intrinsic.[2] These are not to be confused or conflated with other emerging programmed cell death modalities, such as pyroptosis (mediated by Caspase-1 and gasdermin),[3] necroptosis (mediated by the RIP kinases and the pseudokinase Mlkl),[4] or ferroptosis (mediated by ferrous iron-dependent lipid peroxidation).[5]

The intrinsic apoptosis pathway centers on mitochondria (Figure 1). It is governed by the BCL-2 family of proteins. Prosurvival BCL-2 proteins (BCL-2, BCL-X_L, MCL-1, BCL-W, and A1) maintain cellular viability by directly and indirectly restraining prodeath BAK and BAX, the 2 proteins whose activity define the pathway. Upon exposure to stress or developmental cues, the prosurvival signal is overwhelmed, allowing BAK and BAX to become active, whereupon they oligomerize in the mitochondrial outer membrane and form pores, releasing mitochondrial constituents, including cytochrome *c*. The latter triggers the apoptotic caspase cascade, which begins with the initiator caspase, Caspase-9, and culminates in the activation of the effectors, Caspase-3 and Caspase-7. These 2 enzymes cleave hundreds (potentially thousands) of intracellular substrates, resulting in DNA damage, suppression of transcription and protein translation, and the disabling of many other essential cellular processes.[6]

The extrinsic apoptosis pathway is initiated by secreted "death ligands" of the tumor necrosis factor superfamily, such as FasL, which bind to the extracellular domain of death receptors (CD95 [or Fas], in the case of FasL), thereby stimulating the formation of the death-inducing signaling complex (Figure 1). The death-inducing signaling complex facilitates proteolytic cleavage and activation of Caspase-8, which can kill so-called type 1 cells (eg, lymphocytes) directly by triggering Caspase-3/7.[7] In type 2 cells (eg, hepatocytes), the additional recruitment of the intrinsic pathway is required to induce killing. This is achieved via Caspase-8–mediated cleavage of the protein BID, which then directly triggers BAK/BAX activation.[8,9]

BAK and BAX deliver the lethal blow

Loss of BAK and BAX renders cells resistant to a wide range of apoptotic stimuli, exhibiting neither cytochrome *c* release nor caspase activation.[10-14] Most importantly, BAK/BAX-deficient cells are able to not only survive in the short term but also divide and produce viable daughter cells. In contrast, despite having long been thought essential for apoptotic cell death, recent work indicates that the proteolytic cleavage of substrates by Caspase-3 and Caspase-7 is required to prevent an apoptotic cell from tripping its own innate immune sensors.[15,16] BAK/BAX-mediated mitochondrial damage results in the release of mitochondrial DNA, which, in the absence of active caspases, activates the cGAS/STING type I interferon pathway. Accordingly, genetic or pharmacological inhibition of Caspase-9 or Caspase-3/7 allows dying cells to survive long enough to produce interferon-β, but cannot ultimately prevent BAK/BAX-induced death.

Megakaryocytes and MCL-1 and BCL-X_L: staying alive

Like many blood cells, the development, maturation, and survival of megakaryocytes depend on effective restraint of the intrinsic apoptosis pathway (Figure 2). In megakaryocytes, the BCL-2 prosurvival proteins MCL-1 and BCL-X_L fulfill the role.[17,18] This is best illustrated in mice with a *Platelet factor 4 Cre*–mediated megakaryocyte-specific deletion of MCL-1. These animals have no defect in thrombopoiesis and exhibit normal platelet counts. However, treatment with a single dose of ABT-737, a small molecule inhibitor of BCL-X_L (and BCL-2), triggers the rapid-onset apoptotic death of megakaryocytes, which display striking nuclear pyknosis and cytoplasmic dysmorphology.[18] Combined *Pf4-Cre*–mediated deletion of MCL-1 and BCL-X_L results in

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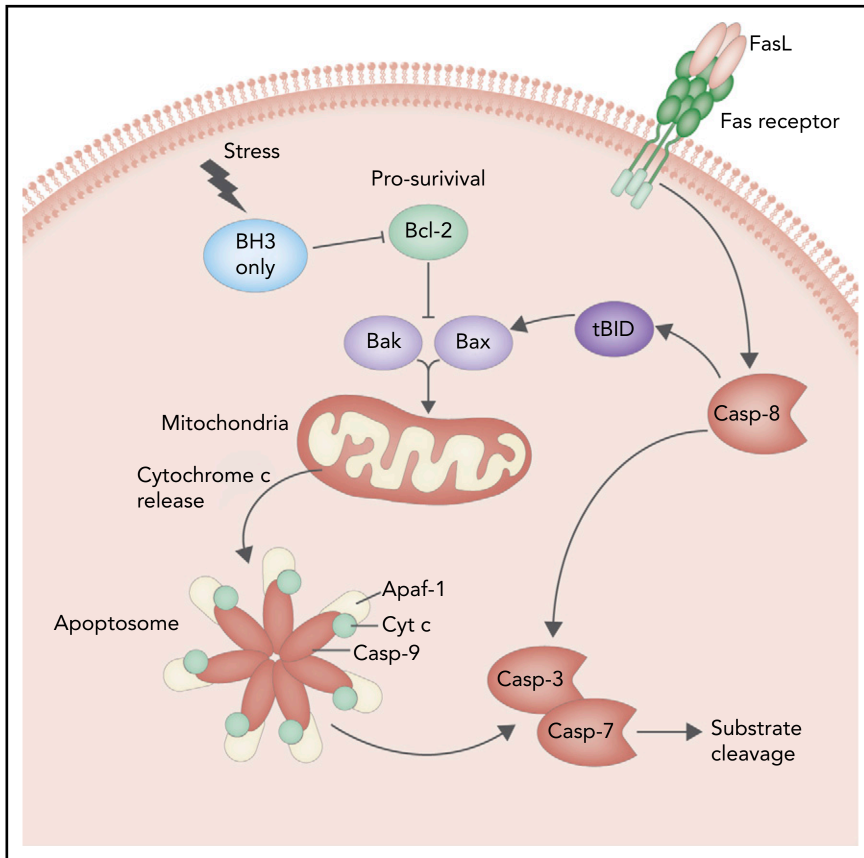

Figure 1. The intrinsic apoptosis pathway. In a healthy cell, prosurvival members (BCL2, BCL-XL, BCL2L2, MCL1, and BCL2A1) restrain the activity of prodeath BAK and BAX. Stress signals activate the BH3-only proteins, which overwhelm prosurvival proteins, thereby activating BAK and BAX. The latter oligomerizes and inserts into the outer mitochondrial membrane, causing permeabilization and release of cytochrome *c* from the mitochondria. This triggers formation of the apoptosome and subsequent activation of caspase-9, and the rest of the apoptotic caspase cascade. The extrinsic apoptosis pathway is induced upon ligand binding to death receptors (eg, FAS ligand [FasL] to FAS receptor). This initiates recruitment of adaptor proteins to the death receptor intracellular domain (death domain), which results in cleavage and activation of caspase-8. Active caspase-8 can directly activate the effector caspases, caspase-3 and caspase-7. In addition, it also can trigger the intrinsic pathway by cleaving BID to produce tBID, which then activates BAK and BAX. Professional illustration by Somersault18:24.

embryonic death, with embryos exhibiting hemorrhages and blood-filled lymphatic vessels.

Therefore, in order to develop, mature, and survive through proplatelet formation and release, BAK/BAX-mediated apoptosis must be restrained. This contrasts with the idea that apoptosis is *required* for platelet biogenesis. Beginning with the observation that in culture systems, peak platelet production by primary mature megakaryocytes "corresponded to the onset of apoptosis,"[19] a body of evidence emerged to support a model whereby mature megakaryocytes undergo apoptosis, "localized" or "para-apoptosis" to facilitate the cytoskeletal rearrangements required for platelet shedding.[20-27] Both the intrinsic and the extrinsic pathways have been implicated, as have the apoptotic caspases: Debili and colleagues reported the presence of active apoptotic caspases in the cytoplasm of cultured primary human CD34^{+}-derived megakaryocytes and showed that proplatelet formation was impaired in the presence of fluoromethylketone caspase inhibitors such as Z-VAD.fmk.[24] However, more recent work using mouse models lacking BAK, BAX, and Caspase-8, or Caspase-9, found no defects in platelet production.[17,28-32] Although some of the discrepancies between the 2 schools of thought can be explained by differences between in vitro and in vivo settings and the use of caspase inhibitors (which, as discussed above, can trigger innate immune signaling within dying cells), the potential "nonapoptotic" roles of classical intrinsic apoptosis components remain to be fully explored.

Once the megakaryocyte has fulfilled its raison d'être, however, it appears the intrinsic apoptosis pathway is deployed. Radley and Haller were the first to make the connection.[33] They reported "degenerate senescent" megakaryocytes in the bone marrow of mice, characterized by "naked" nuclei with a thin ring of cytoplasm. The ultrastructural features of these cells were consistent with death by apoptosis, leading the authors to suggest that, once platelet shedding has occurred, megakaryocytes undergo apoptosis and phagocytosis by macrophages. This hypothesis is supported by observations that mice with a constitutive deletion of BAK and a megakaryocyte-specific deletion of BAX exhibit a fourfold increase in the number of naked nuclei at steady state.[29] Although the modest scale of the change indicates an impairment in the rate at which spent megakaryocytes are cleared from the marrow, rather than an absolute block, the intrinsic apoptosis pathway appears to contribute to the efficient disposal of megakaryocyte nuclei in vivo. Presumably the cytoplasm that remains postshedding contains enough mitochondria to allow apoptosis to proceed.

Interestingly, BAK/BAX-deficient megakaryocytes were significantly resistant to lymphocytic choriomeningitis virus–induced death during chronic infection in mice.[28] This indicates that pathophysiological stresses can indeed trip the apoptotic switch in megakaryocytes, contributing to decreased platelet counts. Apoptotic features have been observed in megakaryocytes in many clinical settings associated with thrombocytopenia, including myelodysplastic syndrome,[34] myeloablative chemo- and radiotherapy,[35] immune-mediated thrombocytopenia,[21] arenaviruses,[36] HIV,[37] dengue,[38] and anthrax.[39] It remains to be established how relevant the intrinsic apoptosis pathway is in each of these contexts, and whether its activation is critical or incidental to loss of megakaryocyte viability.

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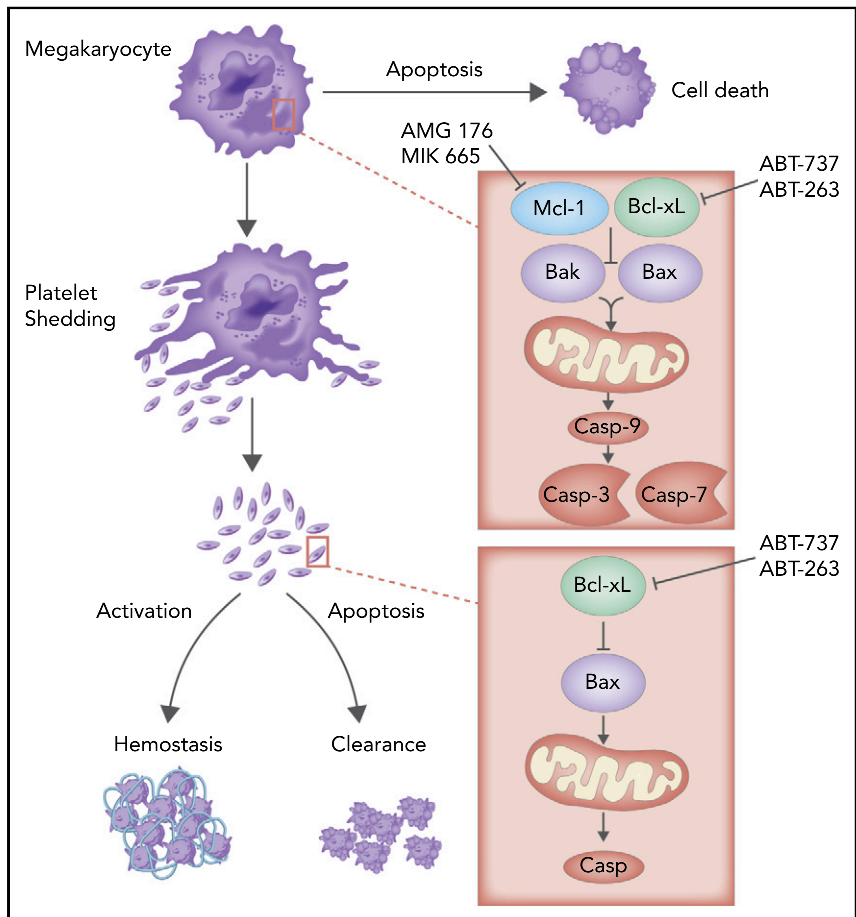

Figure 2. The role of apoptosis in megakaryocyte and platelet biology. Megakaryocytes possess functional BAK/BAX-mediated intrinsic and FasL-inducible extrinsic apoptosis pathways. Both pathways must be restrained during megakaryocyte growth and development in order to allow platelet production to occur. Megakaryocyte apoptosis can be triggered in response to pathophysiological stresses such as chemotherapy or infection. Once shed into the circulation, platelet lifespan is regulated by the intrinsic apoptosis pathway. Bcl-xL is the essential mediator of platelet survival. BH3 mimetic drugs, such as navitoclax (ABT-263), that target Bcl-xL cause platelet apoptosis and thrombocytopenia. Platelets appear to lack the Fas receptor, and the role of Caspase-8 and the extrinsic pathway in platelets is yet to be established. Professional illustration by Somersault18:24.

Platelet survival and lifespan

Platelets enjoy a brief life in the circulation before they are cleared by the reticuloendothelial system: 8 to 10 days in humans,[40] 4 to 5 in mice.[41] This is not a function of their being anucleate; erythrocytes have a circulating lifespan in humans of ~3 months.[42] Although the presence of BCL-2 family members and caspases in platelets was first reported 20 years ago,[43-46] it is only in the last decade that the central importance of the intrinsic apoptosis pathway to their survival has become apparent. An important contributor to this advance has been advent of the "BH3 mimetics," a new class of anticancer drugs that specifically target BCL-2 prosurvival proteins to induce apoptosis in tumor cells. The first bona fide example, the preclinical candidate ABT-737 (which inhibits BCL-2, BCL-X_L, and BCL-W),[47] was found to cause BAK/BAX-mediated platelet apoptosis and, as a result, acute thrombocytopenia, in mice and dogs.[48,49] The critical determinant of platelet survival is BCL-X_L.[49] Its genetic deletion results in dose-dependent reductions in platelet lifespan and subsequent platelet count.[29,31,49] Conversely, combined deletion of BAK/BAX results in a doubling of platelet lifespan and concomitant thrombocytosis.[49] It also renders platelets refractory to ABT-737.

The identification of BCL-X_L as an essential mediator of platelet survival casts new light on the development of the BH3 mimetics. The initial focus of such efforts was BCL-2, a prime target in lymphocytic leukemias and lymphoma, long known to be associated with aberrant expression of this protein.[50,51] When ABT-263 (an orally available analog of ABT-737) entered phase 1 trials, it was anticipated that thrombocytopenia would be an on-target side effect. This proved to be the case, limiting the efficacy achievable in this disease.[52-54] Undaunted, the inventors of ABT-263 reengineered the molecule to create a highly specific inhibitor of BCL-2 called ABT-199.[55] This drug exhibited significant potency against BCL-2–dependent tumors in vivo, without killing platelets. Renamed Venetoclax, ABT-199 moved rapidly through clinical trials,[56] and in April 2016, was approved by the US Food and Drug Administration for the treatment of chronic lymphocytic leukemia with 17p deletion. It is currently in >50 trials for a range of hematological malignancies. Testament to the progress being made in the era of targeted therapies, ABT-263 is now being investigated in myelofibrosis in combination with the Jak2 inhibitor ruxolitinib (NCT03222609). This drug pairing effectively induces apoptosis in human megakaryoblastic JAK2^{V617F} SET-2 cells with acquired resistance to ruxolitinib.[57] It will be interesting to see how this translates in patients with mutant Jak2-driven disease, and whether on-target platelet killing proves beneficial in this setting.

What is the signal for physiological platelet apoptosis?

How each individual platelet decides (or is instructed) to commence apoptosis and trigger clearance from the bloodstream remains a mystery. For many years, the curtailment of platelet lifespan was thought to be the result of accumulated cellular

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damage inflicted by "multiple hits" suffered during circulation.[58] The identification of BCL-X_L as an essential determinant of platelet survival suggested another, cell-intrinsic mechanism, termed the "molecular clock," which holds that BCL-X_L levels decline relative to BAK/BAX until their activation cannot be prevented.[49] More recent work, however, found no change in BCL-X_L levels in aged platelets.[59] If, therefore, apoptosis is not simply the result of metronomic BCL-X_L degradation, then something must activate BAK and BAX directly. The most obvious candidates are the so-called BH3-only prodeath proteins, a subfamily of 8 BCL-2 relatives (eg, BID, BIM, BAD).[2] In nucleated cells, BH3-only proteins are activated in response to proapoptotic signals, whereupon they disrupt the interactions between BCL-2 prosurvivals and BAK/BAX, allowing the latter to become active. As the name suggests, BH3 mimetic drugs function as small molecule versions of BH3-only proteins.

In some cell types, BH3-only proteins are reportedly essential for activation of BAK and BAX.[60] The primary piece of evidence that they contribute to the regulation of platelet survival is that BAD knockout mice exhibit a small but significant extension of platelet lifespan.[61] Several studies have suggested that PI3K/AKT-mediated inactivation of BAD protects platelets from ABT-737 and promotes their survival in vivo.[62-64] This is reportedly downstream of the $P2Y_{12}$ and CXCR7 receptors. BAD has also been suggested to be a substrate of protein kinase A, with downregulation of protein kinase A allowing BAD to initiate platelet apoptosis by sequestering BCL-X_L.[65] These observations indicate that numerous external signals can influence platelet lifespan. They also suggest that the multiple hit model remains relevant today. However, definitive genetic evidence is lacking. The effect of BAD deletion is modest and may simply be the result of removing a sink for BCL-X_L prosurvival activity, not because BAD actively signals to BAK and BAX. If BH3-only proteins are indeed active mediators of platelet apoptosis, then, because loss of BAD does not result in the dramatic extension of lifespan seen in the absence of BAK/BAX, other family members must contribute. In what combination they function and how they integrate intra- and extracellular cues remains to be established.

Platelet activation and apoptosis: joined at the mitochondria

Purified platelets treated with ABT-737 undergo BAK/BAX-mediated mitochondrial damage (cytochrome *c* release, loss of mitochondrial potential, and ATP production), caspase activation, cytoplasmic condensation, and phosphatidylserine (PS) exposure.[48,49,66] These are all hallmarks of apoptosis in nucleated cells. In platelets, they have also been implicated in functional responses triggered by classical agonists. Activated platelets mobilize PS to promote formation of the tenase complex and thrombin generation.[67,68] Similarly, ABT-737–treated platelets can stimulate the formation of thrombin.[66] However, as first postulated by Wolf and colleagues almost 20 years ago,[43] it appears that platelet activation and apoptosis are distinct cellular events. Platelet activation requires calcium, operates at least partly through the action of the mitochondrial matrix protein cyclophilin D, and is independent of BAK/BAX and caspases.[66,69] Conversely, ABT-737-induced PS exposure occurs in the presence of calcium chelators and is blocked by caspase inhibitors. Thus, platelets have evolved 2 pathways to PS exposure. This is backed up by the identification of critical mediators of phospholipid translocation, the so-called scramblases by Nagata and colleagues. The first of these, Tmem16F, is calcium dependent and is mutated in the human bleeding disorder Scott syndrome.[70] It is not required for apoptotic PS exposure. The second protein is Xkr8, which is activated via caspase-3 cleavage during apoptosis and, at least in some cell types, operates independently of calcium.[71]

Whether or not there is cross-talk between the 2 platelet PS mobilization pathways remains to be established. There are many reports of apoptotic-like or apoptosis-associated phenomena in platelets treated with various agents, exposed to shear, or isolated from patients with conditions such as chronic uremia or immune thrombocytopenia.[72] Do highly activated platelets undergo apoptosis as a means to enhance their procoagulant activity? Can pathophysiological stresses induce BAK/BAX-mediated death? Despite the plethora of published observations, it is difficult to ascertain whether classical agonists can activate BAK and BAX, and whether intrinsic apoptosis is required for the observed platelet response. This is primarily due to there being no specific surrogate marker for activation of the intrinsic pathway, and as yet, despite promising developments, no potent, well-characterized chemical inhibitors of BAK and BAX.[73,74] Very few studies of BAK/BAX-deficient platelets have been conducted to date. What is clear, however, is that drugs like ionomycin that can induce loss of mitochondrial potential, caspase activation, and PS exposure in platelets, do so whether or not BAK and BAX are present.[66] Thus, caution should be exercised when interpreting the results of any study where 1 or more of the "hallmarks" of apoptosis are observed.

This point is underscored by a recent study of pulmonary thrombosis following gut ischemia in mice.[75] In this model, neutrophil macroaggregates bound by membrane fragments torn from PS-positive "dying" platelets formed occlusive thrombi in the pulmonary vasculature. To determine whether ischemia-induced PS exposure was driven by apoptosis, mice with a megakaryocyte-specific deletion of BAK and BAX were examined. No protection was afforded. In striking contrast, animals carrying a megakaryocyte-specific deletion of cyclophilin D exhibited an ~80% reduction in neutrophil aggregate formation in mesenteric veins after intestinal ischemia/reperfusion injury. This work reinforces the notion that platelet activation is, ultimately, a form of programmed cell death: "regulated necrosis" initiated by high, sustained levels of cytosolic Ca^{2+} that leads to cyclophilin D–mediated mitochondrial dysfunction and PS exposure.[69,76,77] How platelet necrosis and apoptosis might intersect remains an intriguing question. It may be that BAK/BAX-mediated apoptosis serves no function beyond maintaining platelet survival during circulation, and then efficiently inducing the clearance of those platelets that escape hemostatic consumption or other fates.

Conclusions

Our understanding of how megakaryocyte and platelet development and survival is governed has progressed considerably since William Duke made the seminal observation that "platelets are short-lived bodies."[78] Fundamental questions remain, however. It is unclear how proapoptotic signals (other than BH3 mimetic drugs) induce apoptosis in either megakaryocytes or platelets. We do not understand how intrinsic apoptosis

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pathway–induced platelet clearance and that mediated by the hepatic Ashwell-Morell receptor's recognition of desialyated platelets[79] relate to each other. Both mechanisms regulate platelet lifespan, but their relative contributions, and whether they interact or operate independently, remain to be established. Finally, recent evidence indicates that platelets are capable of inducing apoptosis in target cells via membrane-bound Fas ligand.[80] This remarkable observation suggests that in addition to dying by the sword, platelets may also live by it too.

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