News and Commentary

Cell death in the absence of Bax and Bak

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When the Korsmeyer laboratory reported that a B-cell-specific *bcl-2* transgene led to an expansion in murine B-cells by conferring a survival advantage to the cells, it ushered in a new era in cancer research.[1] This paper complemented a finding published previously by the Adams' laboratory showing that overexpression of *bcl-2* in growth factor-dependent cells could keep these cells alive following growth factor withdrawal *in vitro*.[2] These two observations initiated the study of the role of cell death regulation in the pathogenesis and treatment of cancer. The early years of apoptosis research saw the cloning of a number of *bcl-2*-related genes that appear to play key roles in the regulation of programmed cell death (apoptosis).[3] Surprisingly, this *bcl-2*-related family of genes contained both proapoptotic and antiapoptotic members. The roles of these genes in development and tissue homeostasis in the mouse have been delineated in large part owing to the techniques of targeted gene deletion and tissue-specific transgenesis. Collectively, these studies suggest that the Bcl-2 family regulates the ability of damaged and/or excess cells to be deleted from the host by apoptosis. Overexpression of antiapoptotic family members can act as oncogenes by promoting the survival of partially transformed cells. In contrast, the proapoptotic family members appear to have evolved to suppress cell autonomous survival in adult animals. The proapoptotic family members consist of Bax and Bak, which show ubiquitous tissue expression, Bok, which is mainly expressed in reproductive organs.[4] Two other proapoptotic family members, Bid[5] and BCL2L13,[6] which share overall homology to other Bcl-2 family members appear to function only as initiators of Bax- and /or Bak-dependent killing. The targeted deletions of the two major proapoptotic genes *bak* and *bax* produced mice with no overt phenotype[7] or with a relatively mild phenotype, respectively.[8] However, when both *bax* and *bak* were deleted, the resulting mouse was found to have a dizzying array of defects.[7] This commentary describes the major phenotypes of the *bax*$^{-/-}$*bak*$^{-/-}$ mouse and considers cell survival and cell death in the absence of Bax and Bak.

Development in the Absence of Bax and Bak

During development, the interdigital mesoderm undergoes apoptosis so that by embryonic day 15.5, the separation of the digits in mice has been completed. A number of genes have been implicated in this form of cell death.[9] However, the individual contribution of these genes can be hard to evaluate either because the interdigital tissue is ultimately resorbed or the embryos die before birth.[10,11] In contrast, as shown in Figure 1a, the *bax*$^{-/-}$*bak*$^{-/-}$ mice display the persistence of interdigital webs into adulthood.[7] This phenotype is observed in all *bax*$^{-/-}$*bak*$^{-/-}$ mice. This places Bax and Bak as central determinants of the developmental removal of the interdigital tissue. Defects in developmental apoptosis are also seen in other tissues. Excess cells are observed in the central nervous system and most embryos die at or just before birth. Female *bax*$^{-/-}$*bak*$^{-/-}$ mice that survive until adulthood display imperforate vaginas.[7] In addition, fetal ocular vasculature persists in the adult *bax*$^{-/-}$*bak*$^{-/-}$ mice.[12] Thus, the proapoptotic activities of Bax and Bak play an integral role in the shaping of tissues during development. Despite this, some *bax*$^{-/-}$*bak*$^{-/-}$ mice survive development and live into adulthood (Figure 1a).

Bax and Bak Controls the Apoptotic Machinery

Although Bax and Bak share an overall structural homology with the antiapoptotic Bcl-2-related proteins, there exists a second group of proapoptotic proteins, the so-called BH3-only proteins, which contain only a single domain of homology with Bcl-2. These proteins/genes are inactive in viable cells as a result of post-translational modifications or transcriptional repression. One early insight gained from the study of cell lines from the *bax*$^{-/-}$*bak*$^{-/-}$ mice came from experiments aimed at defining the proapoptotic functions of BH3-only proteins. When the proapoptotic form of the BH3-only protein Bim (BimS) was introduced into *bax*$^{-/-}$*bak*$^{-/-}$ mouse embryo fibroblasts (MEF), the cells remained viable whereas single *bax*$^{-/-}$, *bak*$^{-/-}$, or wild-type cells died rapidly.[13] The introduced BimS was functional as it was able to co-precipitate both endogenous Bcl-2 and Bcl-x$_L$. Similar results were obtained following transfection with Bad and Noxa.[13,14] These data demonstrated that inhibitors of antiapoptotic Bcl-2 proteins do not promote cell death in the absence of Bax and Bak. Similarly, a second group of BH3-only proteins such as tBid, defined by the ability to bind Bax and Bak, is also incapable of initiating apoptosis in *bax*$^{-/-}$*bak*$^{-/-}$ cells.[15] Furthermore, *bax*$^{-/-}$*bak*$^{-/-}$ MEF became as susceptible as wild-type cells when Bax and BH3-only proteins were introduced at the same time. *bax*$^{-/-}$*bak*$^{-/-}$ MEF also became sensitive to other death-inducing agents such as etoposide, staurosporine, brefeldinA, and UV treatment, when Bax was
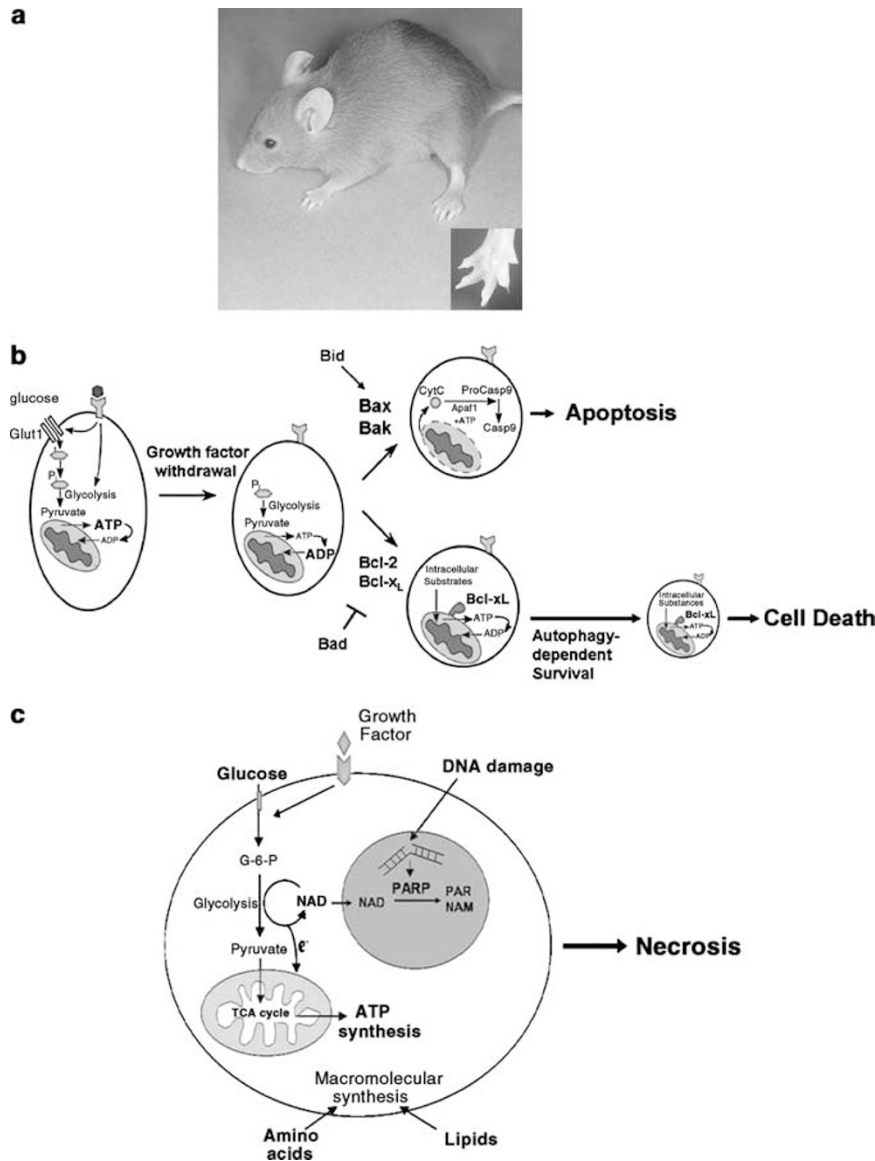
Figure 1 (**a**) Despite a high perinatal mortality, some *bax*$^{-/-}$*bak*$^{-/-}$ mice reach adulthood displaying multiple defects in developmental apoptosis such as persistent interdigital webs. (**b**) Regulation of cell fate following growth factor withdrawal in the presence or absence of Bax and/or Bak. To maintain the uptake of nutrients, growth factor-dependent cells rely on signaling events initiated at the growth factor receptor. When growth factor is withdrawn, the cell shrinks and is eliminated by apoptotic cell death, which depends on the proapoptotic activity of Bax and/or Bak. The proapoptotic activity of Bax and/or Bak can be either directly induced by BH3 proteins like Bid or secondarily released by BH3 inhibitors, like Bad, that suppress the antiapoptotic activity of antiapoptotic Bcl-2 proteins like Bcl-2 or Bcl-x$_L$. In the absence of Bax and Bak, or as a result of over expression of Bcl-2 or Bcl-x$_L$, apoptosis is suppressed and the cells will turn to autophagy for survival, which however is limited, and the cell will in time die from a bioenergetic collapse (**c**) DNA damage induces PARP-dependent necrosis in proliferating cells. Growth factor-dependent cells generate ATP by aerobic glycolysis. DNA damage activates the enzyme PARP, which depletes cytosolic NAD pools making the cell unable to degrade glucose to support ATP production and the cell undergoes necrotic cell death

re-introduced. These experiments demonstrated that Bax and Bak are required for the initiation of apoptotic cell death. In the absence of Bax and Bak, BH3-only proteins are not able to induce cell death. Thus, in a healthy, viable cell, the proapoptotic proteins Bax and Bak are kept in check by the antiapoptotic molecules Bcl-2 and Bcl-x$_L$ and the BH3-only proteins are inactive. When the cell is subjected to death-inducing stimuli such as DNA damage, growth factor deprivation, or ER stress, the BH3-only proteins are induced. Depending on the individual BH3-only protein, it can either bind to an antiapoptotic Bcl-2 protein and inhibit their activity or bind to Bax and/or Bak to induce their effector function. Either action results ultimately in the loss of the integrity of the outer mitochondrial membrane and apoptotic cell death.

Cell Survival in the Absence of Bax and Bak

A striking abnormality of *bax*$^{-/-}$*bak*$^{-/-}$ adult mice is the progressive accumulation of non-proliferating lymphocytes as the animals age.[7] Not only do these lymphocytes accumulate

in lymphoid organs they also infiltrate mesenchymal organs such as the liver and kidneys. Analysis of cell surface markers showed a predominance of memory type cells in both the B-cell and T-cell compartment. The explanation for this accumulation of memory cells was revealed when it was found that $bax^{-/-}bak^{-/-}$ spleen cells placed in culture in the absence of cytokines survived for days, whereas wild-type spleen cells started to die during the first day in culture and all died by 96 h when cultured in the absence of cytokines. This type of apoptosis has been termed death-by-neglect. In the absence of Bax and Bak, cells which would normally die in the absence of survival factors continue to live.

In order to study the mechanism behind this phenomenon, cell lines from multiple tissues of $bax^{-/-}bak^{-/-}$ mice were prepared. In a first set of experiments, MEF were prepared from $bax^{-/-}bak^{-/-}$ and control littermate embryos. The serum was removed from the MEF medium and the viability of the cultures assessed over several days.[15] Whereas the majority of wild-type, $bax^{-/-}$, or $bak^{-/-}$ cells were dead by day 3 following serum removal, the $bax^{-/-}bak^{-/-}$ cultures remained viable. In a parallel set of experiments, the dependence of neural progenitor cells (NPC) on fibroblast growth factor-2 (FGF-2) was examined.[16] The NPC prepared from the brains of neonatal $bax^{-/-}bak^{-/-}$ mice remained alive following removal of FGF-2, whereas the majority of cells in cultures of NPC from wild-type mice or single bax- or bak-deficient mice had died by day 3. An *in vivo* correlate to this phenomenon was observed in the brains of adult $bax^{-/-}$ $bak^{-/-}$ mice, which display enhanced numbers of NPC. It could be hypothesized that these excess NPC would normally be removed by apoptosis, as their accumulation would deplete the local levels of growth factors. However, in the absence of Bax and Bak, these cells are able to survive.

Hematopoietic cell lines dependent on the growth factor IL-3 have been widely studied because of the ease of growing large numbers of these cells in suspension cultures, the ability to transfect them, and the fact that these cell lines are immortalized but not transformed. IL-3-dependent cell lines were produced from bone marrow of $bax^{-/-}bak^{-/-}$ mice.[17] As shown previously for MEF cultures in response to serum withdrawal and NPC cultures in response to FGF-2 removal, IL-3-dependent cells prepared from $bax^{-/-}bak^{-/-}$ mice survive following removal of IL-3 for weeks, whereas control cells die in 2–3 days. The only apparent reason for cell death in this timescale is the induction of apoptosis as Apaf-1- and caspase-9-deficient cells also maintain their viability following growth factor deprivation over this timescale.[18] Within the first few days of IL-3 deprivation, $bax^{-/-}bak^{-/-}$ cells decreased in size by half and the size continued to decrease steadily until after several weeks the cells began to die. When examining the metabolic profile for these cells, it was found that the glycolytic rate rapidly declined following IL-3 withdrawal. Without IL-3, the cells cease to express the GLUT1 transporter. Thus, despite the fact that the cells were surrounded by ample glucose levels in the surrounding media, they were unable to take up sufficient glucose to sustain themselves in the absence of IL-3. However, the ATP levels in these growth factor-deprived cells did not show the same rapid decline as GLUT1 expression. Thus, it appeared that the cells were able to generate ATP for survival by another mechanism. One way

that cells can generate ATP in the absence of exogenous nutrients is through the process of autophagy where internal organelles and cytoplasm are metabolized to provide ATP for cell survival. Indeed, electron microscopy of $bax^{-/-}bak^{-/-}$ cells showed an increase in autophagosomes, vesicles containing intracellular material surrounded by a double membrane, within 48 h of growth factor withdrawal. To confirm that autophagy was taking place, the cells were stained with an antibody specific for the mammalian homolog of the yeast Atg8 protein, microtubule-associated protein-1 light chain-3 (LC3). LC3 associates with the forming autophagosomes and following induction of autophagy, staining will redistribute from a diffuse cytoplasmic staining pattern to a punctate, vesicular staining pattern. $bax^{-/-}bak^{-/-}$ cells, which had been deprived of IL-3 for 2 days, showed a conversion of LC3 staining to a punctate pattern.

To further confirm that autophagy was keeping the $bax^{-/-}bak^{-/-}$ cells alive, the effect of inhibition of autophagy was examined in these cells. Autophagy was inhibited by introducing an shRNA directed against the autophagy gene *Atg5*. Indeed, $bax^{-/-}bak^{-/-}$ cells stably transfected with *Atg5* shRNA died rapidly following removal of IL-3. Similarly, suppression of *Atg7* gave similar results. Autophagy can also be inhibited by two drugs; chloroquine and 3-methyladenine. Treatment of IL-3-deprived $bax^{-/-}bak^{-/-}$ cells with either drug led to rapid cell death. In unicellular organisms, autophagy can keep the organism alive during nutrient deprivation, and when nutrients are resupplied, the cell can recover its normal cellular functions. This was also the case for growth factor-deprived $bax^{-/-}bak^{-/-}$ cells. Within 24 h of re-addition of IL-3 to deprived $bax^{-/-}bak^{-/-}$ cultures, the glycolytic rate was restored to the same level observed before growth factor was removed. The recovery time for cell size and proliferation depended on how long the cells had been deprived of IL-3. Nevertheless, the surviving cells remained responsive to IL-3 and were ultimately able to recover.

In these experiments, the $bax^{-/-}bak^{-/-}$ growth factor-dependent cell lines proved a valuable tool in delineating a cell survival mechanism that was difficult to study in mammalian cells with an intact apoptotic machinery. However, autophagy is a self-limiting survival mechanism and the cells will eventually die from a bioenergetic collapse unless nutrient uptake is restored. A similar role for autophagy in suppressing necrotic death has been established in nutrient-deprived yeast and plant cells.[19,20] Recent independent evidence supports a role for autophagy in organismal survival as well. Kuma *et al.*[21] found that during the first day after birth, a large number of autophagosomes could be observed in multiple organs of newborn mouse pups born to mice that carry a ubiquitously expressed GFP-LC3 transgene. As the pups started to nurse efficiently, the autophagosome count declined. Mice with a targeted deletion of the mammalian *Atg5* gene, which is necessary for autophagosome formation, die during the first day of life before nursing can be established.

Cell Death in the Absence of Bax and Bak

Despite its profound apoptotic defects, $bax^{-/-}bak^{-/-}$ mice can survive well into adulthood (Figure 1a). Even though there

is a very high perinatal mortality for $bax^{-/-}bak^{-/-}$ pups, $bax^{-/-}bak^{-/-}$ mice born alive can live for months, indicating that even in the absence of apoptotic cell death, the tissue homeostasis of most adult organs is maintained. These results suggest that there are other forms of cell death occurring in the absence of Bax and Bak, which can serve to eliminate excess and/or damaged cells during development and tissue homeostasis. This possibility is also suggested by the observation that follicular lymphomas, which have an enhanced expression of the antiapoptotic gene bcl-2, owing to the 14:18 chromosomal translocation, remain sensitive to radiation and chemotherapy.[22] To further characterize this Bax/Bak-independent cell death, the survival of normal and $bax^{-/-}bak^{-/-}$ MEF cell lines has been studied following treatment with a variety of chemotherapeutic agents. $bax^{-/-}bak^{-/-}$ MEF were resistant to treatment with DNA topoisomerase inhibitor etoposide, whereas wild-type MEF die an apoptotic cell death. However, when $bax^{-/-}bak^{-/-}$ MEF were treated with the alkylating agents nitrogen mustard and N-methyl-N′nitro-N-nitrosoguanidine (MNNG), the cells died with the same kinetics as wild-type cells.[23]

DNA alkylation activates the enzyme PARP.[24] PARP acts by synthesizing poly(ADP-ribose) polymers on histones and other chromatin-associated proteins. This process facilitates the recognition of DNA damage by DNA repair enzymes. The substrate for PARP is β-nicotinamide adenine dinucleotide (NAD) and following PARP activation, cytosolic/nuclear NAD but not mitochondrial NAD is depleted.[25,26] As a result of cytosolic NAD depletion, glucose metabolism ceases. In growing cells, which utilize glucose as their major metabolic substrate, cytosolic NAD depletion leads to loss of ATP generation and necrotic cell death. In contrast, in vegetative cells, which maintain the ability to catabolize lipids in their mitochondria, cytosolic NAD depletion has little effect on ATP production and the cells survive to attempt DNA repair. When PARP expression was suppressed by an shRNA, necrotic cell death in response to treatment with MNNG is prevented. The morphology of $bax^{-/-}bak^{-/-}$ cells dying following treatment with DNA alkylating agents reveals cellular swelling and plasma membrane disintegration. No cytochrome c release was seen in the $bax^{-/-}bak^{-/-}$ cells. Necrotic cell death is known to lead to the release of inflammatory molecules, such as high-mobility group box 1 (HMGB1), into the extracellular space.[27] HMGB1 is a ligand for the scavenger receptor RAGE on monocytes/macrophages. It was found that following treatment with MNNG, HMGB1 was translocated from the nucleus to the cytoplasm. Supernatants from MNNG-treated cells elicited an inflammatory response when added to macrophage cultures. Thus, alkylating agents appeared to induce a necrotic cell death and this cell death pathway is intact in cells lacking Bax and Bak. Wild-type cells showed a mixture of both apoptotic and necrotic cell death in response to MNNG.

Alkylating agents have also been shown to induce selective cell death in rapidly dividing cells, whereas non-proliferating cells are spared.[28] The IL-3-dependent hematopoietic $bax^{-/-}bak^{-/-}$ cell lines proved a useful tool to further elucidate this phenomenon. It was found that indeed $bax^{-/-}bak^{-/-}$ cells grown in the presence of IL-3 were sensitive to treatment with MNNG, whereas cells cultured in the absence of IL-3 were resistant to MNNG treatment. The basis for this difference in sensitivity was not differences in PARP activation or NAD depletion, which were similar in both cultures. The difference in survival was owing to the dependence of proliferating cells on glucose for ATP generation. When the medium of proliferating cells was supplemented with a membrane-permeant form of pyruvate, the glycolytic end product, they were no longer dependent on cytosolic NAD and the culture became resistant to MNNG-induced necrotic cell death. This type of experiment was also used to demonstrate that this form of cell death was not peculiar to $bax^{-/-}bak^{-/-}$ cells but also occurred in normal cells. Thus, using the $bax^{-/-}bak^{-/-}$ cell lines, it was possible to dissect a novel, regulated cell death pathway. The properties of this form of death provide an explanation to the long-standing observation that cancer cells, despite having acquired mutations making them resistant to apoptosis, are often acutely sensitive to DNA-damaging drugs.

Conclusion

Cell lines established from $bax^{-/-}bak^{-/-}$ mice have proven an invaluable tool in delineating novel pathways for both cell survival and cell death. As summarized in Figure 1b, cells grown in the presence of permissive growth factors, such as IL-3 for hematopoietic cell lines, will upregulate the GLUT1 transporter, which allows glucose to be taken up by the cell. Glycolysis will then fuel ATP synthesis. However, when growth factor is removed, the GLUT1 transporter is lost from the cell surface and glucose is no longer taken up by the cell. In a normal cell that contains Bax and/or Bak, this rapidly leads to loss of mitochondrial outer membrane integrity and activation of a caspase cascade, hallmarks of apoptotic cell death. However, in the absence of Bax and Bak, the effect of the antiapoptotic molecules like Bcl-2 and Bcl-x_L predominates and the integrity of the outer mitochondrial membrane is maintained and the cell responds to the decline in glucose uptake by initiating autophagy. However, autophagy is a self-limited survival strategy and in time the cell still dies. Thus, the death of neglected cells is not dependent on Bax and/or Bak but rather results from the dependence of the cells on ligand-initiated signal transduction to maintain nutrient uptake at levels sufficient to sustain bioenergetics.

Studies of $bax^{-/-}bak^{-/-}$ cells have also helped establish that necrosis is a form of regulated cell death (Figure 1c). In contrast to apoptosis, necrosis alerts the organism to the need to defend and/or repair itself. The ability of necrosis to initiate such a response results from the specific release of inflammatory mediators and of factors that initiate wound repair. The study of $bax^{-/-}bak^{-/-}$ mice suggests that the field of programmed cell death is much richer and complex than previously appreciated.

Acknowledgements

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