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News and Commentary

Emerging role of Mcl-1 in actively counteracting BH3-only proteins in apoptosis

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Apoptosis is a physiological form of cell death, which plays an essential role during development and in maintaining the homeostasis of multicellular organisms, and its perturbed regulation contributes to many human diseases, including cancer. It occurs in two phases, an initial decision-making or commitment point followed by an execution phase involving many biochemical and morphological changes characteristic of apoptosis. Although the execution phase is mainly mediated by caspases that belong to a family of cysteine proteases, the commitment of cells to apoptosis in response to diverse physiological cues and cytotoxic agents is primarily regulated by proteins of the B-cell lymphoma 2 (Bcl-2) family that is evolutionarily conserved from nematodes to humans. B-cell lymphoma 2 family proteins share one or more Bcl-2 homology (BH) domains and are classified into two main groups, based on their pro- or anti-apoptotic activities.[1,2] The antiapoptotic members include Bcl-2, Bcl-x_L, A1, Bcl-w and Mcl-1. The proapoptotic family members are further divided according to whether they contain multiple BH domains such as Bax and Bak or only the BH3 domain such as Bim and Bid. It has also been proposed that the BH3-only proteins can be re-arranged into two sub-groups – *activating* or *sensitising*.[3,4] Those proteins with *activating* BH3 domains may have a higher affinity for Bax or Bak than Bcl-2 protein (or other antiapoptotic Bcl-2 family members), so perhaps directly activating Bax and Bak.[3,4] The BH3-only proteins with *sensitising* BH3 domains appear to preferentially bind Bcl-2-like proteins allowing Bax and Bak to be de-suppressed and readily activated.

BH3-only Proteins are the Essential Initiators of Apoptosis

Genetic and biochemical studies in mammals and *Caenorhabditis elegans* have shown that the proapoptotic BH3-only proteins are the essential initiators of apoptotic cell death.[5] Gain-of-function mutations in *egl-1*, the BH3-only gene in *C. elegans,* cause the hermaphrodite-specific neurons (HSNs) to undergo programmed cell death, whereas a loss-of-function mutation prevents not only the cell death of HSNs but nearly all normally occurring programmed cell death in somatic cells as well.[6] Egl-1 initiates cell death by binding to Ced-9, the *C. elegans* homologue of Bcl-2, and displacing Ced-4 from the Ced-9/Ced-4 complex, thereby allowing Ced-4 to activate Ced-3, the caspase responsible for the destruction of the cell.[7]

In mammals, there exists at least eight BH3-only proteins,[8,9] making the study of their individual roles in the initiation of apoptosis more complex and difficult. Nevertheless, gene-targeting experiments in mice have begun to reveal their important physiological functions in apoptosis. For example, Bim-deficient mice accumulate excess lymphoid and myeloid cells and die early of mainly autoimmune kidney disease.[10] Bim-deficient lymphocytes are resistant to apoptosis in response to cytokine deprivation, calcium ion flux and microtubule perturbation.[10] Bim is also essential for elimination of the autoreactive thymocytes[11] and B cells.[12] Bim also plays an important, yet undefined, role in embryonic development as the number of $bim^{-/-}$ offspring is less than half that of their wild-type littermates.[10] Bid-deficient mice display resistance to death receptor-induced hepatocyte apoptosis.[13] $Bid^{-/-}$ mice also show increased tumour incidence and reduced survival owing to death from tumour, suggesting that Bid is required for the suppression of certain tumours.[14] It has now been established that BH3-only proteins function at an apical level in an apoptotic signalling cascade that leads to the release of cytochrome *c* from mitochondria and activation of caspases. A variety of seemingly unrelated apoptotic stimuli, including ligation of Fas and related tumour necrosis factor family receptors, deprivation of growth factors, DNA damage and loss of cell matrix attachment, all impinge on the activation of specific BH3-only proteins through transcriptional and post-translational mechanisms.[8,15] Activated BH3-only proteins can either bind to membrane-bound antiapoptotic members to unleash the proapoptotic proteins such as Bax and Bak or perhaps directly interact with and activate these proteins. The BH3-only proteins Bim and the truncated form of Bid (referred to as tBid hereafter) have been shown to directly interact with Bcl-2[16,17] and Mcl-1[18,19] and to antagonise their antiapoptotic activity. In addition, both tBid and some forms of Bim could bind to and activate Bax and/Bak, triggering the oligomerisation of these proteins in mitochondrial membranes, resulting in cytochrome *c* release into the cytoplasm.[20–24] Importantly, in double $Bax^{-/-}$ $Bak^{-/-}$ cells, both tBid and Bim fail to induce cytochrome *c* release and apoptosis, suggesting that they act upstream of and depend upon Bax and/or Bak to exert their proapoptotic effects.[25,26]

It must, however, be emphasised that, although BH3-only proteins require Bax and/or Bak to deliver their apoptotic functions, it still remains unclear whether they activate Bax or Bak through direct protein–protein interaction in intact cells as the evidence for binding of BH3-only proteins to Bax or Bak has so far been inconsistent.[9] Further structural and

biochemical studies on the characteristics of interaction, if any, between BH3-only proteins and Bax or Bak are required to establish the definitive nature of interaction between these proteins.

Recent studies have also demonstrated a role for BH3-only proteins in tumour suppression. In addition to Bim and Bid described above, Puma has been identified as a potent tumour suppressor. The silencing of *puma* in haematopoietic stem cells derived from *myc* transgenic mice by RNAi accelerates lymphomagenesis in recipient mice.[27] *Bad*-deficient mice develop diffuse large B cell lymphoma of germinal centre origin.[28] Clinical studies have also begun to show that BH3-only proteins may act as tumour suppressors in many forms of human cancers. For example, a consistent loss of Bik expression is observed in primary renal cell carcinoma as a result of deletion of the *bik* gene at 22q13.2 locus.[29] Among mantle cell lymphoma patients, 17% cases display homozygous deletion of *bim* gene at 2q13.[30]

Activation of BH3-only Proteins

At least four mammalian BH3-only genes have been shown to be subject to transcriptional control, including *hrk/dp5*, *noxa*, *puma/bbc3* and *bim*. For example, growth factor withdrawal induces the expression of *bim* and *hrk/dp5* in neonatal sympathetic neurons[31–33] and also results in an increased expression of Bim in haematopoietic cells.[34,35] In response to DNA damage, both Noxa and Puma/Bbc3 mRNA and protein levels are upregulated in a p53-dependent manner.[36–39]

Other BH3-only proteins are present in latent forms in healthy cells and their activation requires post-translational modifications. In cells that are stimulated with certain growth factors, Bad is phosphorylated at multiple serine residues, resulting in its sequestration in the cytoplasm by binding to 14-3-3 scaffold proteins.[40] Growth factor deprivation results in accumulation of de-phosphorylated Bad, which, upon release from the complex with 14-3-3, translocates to intracellular membranes where de-phosphorylated Bad binds to Bcl-2 and Bcl-x_L, resulting in the displacement of Bax from Bcl-2/Bax and/or Bcl-x_L/Bax complexes.[40] In addition to the transcriptional regulation, Bim is also subject to post-translational activation. Bim protein is expressed in many tissues and kept inactive in some cell types by sequestration to microtubular complexes, where it binds to a component of the dynein motor complex, dynein light chain LC8.[41] Certain stress conditions, such as UV irradiation, treatment with taxol or cytokine withdrawal, induce the release of Bim from the dynein motor complex, allowing Bim to translocate to mitochondrial membranes, where it binds to and neutralises antiapoptotic Bcl-2 family members,[41] or directly activates other proapoptotic proteins.[24] Full-length Bid is relatively inactive in unstressed cells. However, after cleavage by caspase-8 as a result of ligation of death receptors such as Fas[42,43] or by granzyme B released from cytotoxic T lymphocytes,[44] cleaved Bid (tBid), further modified by post-proteolytic N-myristoylation,[45] translocates to mitochondria to induce oligomerisation of Bax and/or Bak and cytochrome c release.[20,21] This function of tBid is vital for the transmission of apoptotic signals from death receptors to mitochondria in certain tissues and, indeed, apoptosis in hepatocytes is dependent on tBid-mediated amplification of the apoptotic signal via the mitochondria after engagement of the death receptor Fas.[13] tBid can also bind to Bcl-2,[16,46] A1,[47] Bcl-x_L[46] and Mcl-1[19,48] and antagonise their antiapoptotic activity. Bim and tBid thus appear to be the unique BH3-only proteins perhaps capable of both *activating* and *sensitising* other proapoptotic Bcl-2 family proteins. Other BH3-only proteins subject to post-translational modification include Bmf[49] and Bik.[50]

Biological Functions of Mcl-1

Mcl-1 was initially discovered as a gene induced early during differentiation of the myeloid cell line, ML-1.[51] It is expressed in a variety of human tissues and cells as well as many tumours. The fundamental genetic studies in mouse that have shed great light on the functions of Mcl-1 have all been carried out in the laboratory of Professor Stanley Korsmeyer. Deletion of *Mcl-1* in mice leads to embryonic lethality owing to a failure of implantation of embryos in the uterus, suggesting that it is essential for embryonic development.[52] This is in contrast with Bcl-2, which does not play a predominant role during early development.[53,54] Conditional knockout studies reveal that *Mcl-1* is required both in early lymphoid development and in the maintenance of mature B and T lymphocytes, which are rapidly lost when *Mcl-1* is deleted.[18] It is also required for the survival of haematopoietic stem cells[55] and myeloid cells.[56] The expression of Mcl-1 can be upregulated by a number of cytokines, including granulocyte-macrophage colony-stimulating factor, stem cell factor and interleukin (IL)-3, IL-5, IL-6, IL-7 and IL-15 in various haematopoietic cells. In addition, Mcl-1 is subject to post-translational modification. For example, activation of protein kinase C by 12-*O*-tetradecanoylphorbol 13-acetate induces phosphorylation of Mcl-1, resulting in stabilisation of the normally rapidly degraded Mcl-1 protein.[57] Its rapid upregulation in response to cytokines, together with the fact that, unlike Bcl-2, Mcl-1 is a short-lived protein with a half-life between 30 min and a few hours depending on the cell types and culture conditions,[58,59] make Mcl-1 ideally suited to provide short-term protection against cell death during critical transitions in cell fate.[60]

However, enhanced Mcl-1 expression also contributes to a malignant phenotype in certain tumour cells. Transgenic mice overexpressing Mcl-1 display a high rate of B-cell lymphoma.[61] Increased expression of Mcl-1 is also observed in human B-cell chronic lymphocytic leukaemia cells[62] and in bone marrow cells from patients with acute myelogenous and acute lymphocytic leukaemia at the time of relapse.[63] Mcl-1 is also an important survival factor for multiple myeloma. Increased expression of Mcl-1 is required for IL-6-dependent survival of many multiple myeloma cell lines.[64] Downregulation of Mcl-1 using antisense oligonucleotides has been shown to induce apoptosis in these cells.[64] Deregulated expression of Mcl-1 can therefore have a profound effect on cell growth and survival.

Mcl-1 Binds to and Counteracts BH3-only Proteins

Recent studies have demonstrated that Mcl-1 functions as an early critical regulator in the apoptosis signalling that leads to the activation of the mitochondrial pathway. Mcl-1 has been shown to directly interact with the BH3-only proteins Bim[18,65] and tBid,[19,48] and to inhibit their induction of cytochrome *c* release in isolated mitochondria and apoptosis.[48,65] In addition, both Bim and tBid appear to have a higher affinity for Mcl-1 than Bcl-2 in *in vitro* binding experiments,[18,19] suggesting that Mcl-1 may counteract *activating* BH3-only proteins more actively than Bcl-2 in certain cells. This early critical role for Mcl-1 is supported by the significant finding that Mcl-1 functions upstream of and together with Bcl-x_L in preventing UV irradiation-induced cytochrome *c* release from mitochondria and apoptosis in HeLa cells.[66] As mentioned earlier, in response to DNA damage, two BH3-only proteins Noxa and Puma are induced in a p53-dependent manner. Gene deletion of either *puma* or *noxa* leads to severe impairment of the induction of apoptosis by γ-irradiation, DNA-damaging drugs and growth factor withdrawal,[67–69] suggesting that both Noxa and Puma are required for DNA damage-induced apoptosis. Recent studies using *in vitro* binding assay showed that Mcl-1 can bind to both Noxa and Puma with high affinity and inhibit their proapoptotic activity.[70,71] In response to DNA damage by γ-irradiation, etoposide as well as UV irradiation, Mcl-1 disappears early during the induction of apoptosis.[66] The degradation of Mcl-1 has been shown to be mediated by an E3 ubiquitin ligase, Mule, through the proteasome pathway.[72] Interestingly, Mule also contains a BH3 domain. Elimination of Mcl-1 may possibly free these BH3-only proteins to activate Bax and/or Bak leading to the release of cytochrome *c* from mitochondria and activation of caspases. The idea is also consistent with the observation that in adenoviral protein E1A-induced apoptosis in HeLa cells, loss of Mcl-1 is required to initiate the apoptotic pathway.[73] Mcl-1 has also been shown to interact with the proapoptotic Bcl-2 family protein Bak in healthy, unstressed cells,[19,73,74] probably keeping Bak in check. Indeed, a recent study has shown that Bak is kept inactive as a result of forming a complex with Mcl-1 and Bcl-x_L in healthy cells.[75] It induces apoptosis only when displaced from these complexes by Noxa. These data demonstrate that Mcl-1 functions at multiple sites in the apoptotic pathway as an antiapoptotic molecule (Figure 1), therefore playing a more active role in protecting cells from apoptosis than initially thought.

Role of Mcl-1 Cleavage in Apoptosis

Cleavage of Mcl-1 was first reported during Fas-mediated apoptosis of human leukaemic Jurkat T cells.[76] This was later confirmed to occur during apoptosis induced by a variety of stimuli in diverse cell systems.[59,77–79] Site-directed mutagenesis of human Mcl-1 reveals two aspartate residues at positions 127 and 157 in the sequences EEL**D**′G and TST**D**′G, respectively, as the caspase cleavage sites (Figure 2a).[59,79] The cleavage patterns are also confirmed

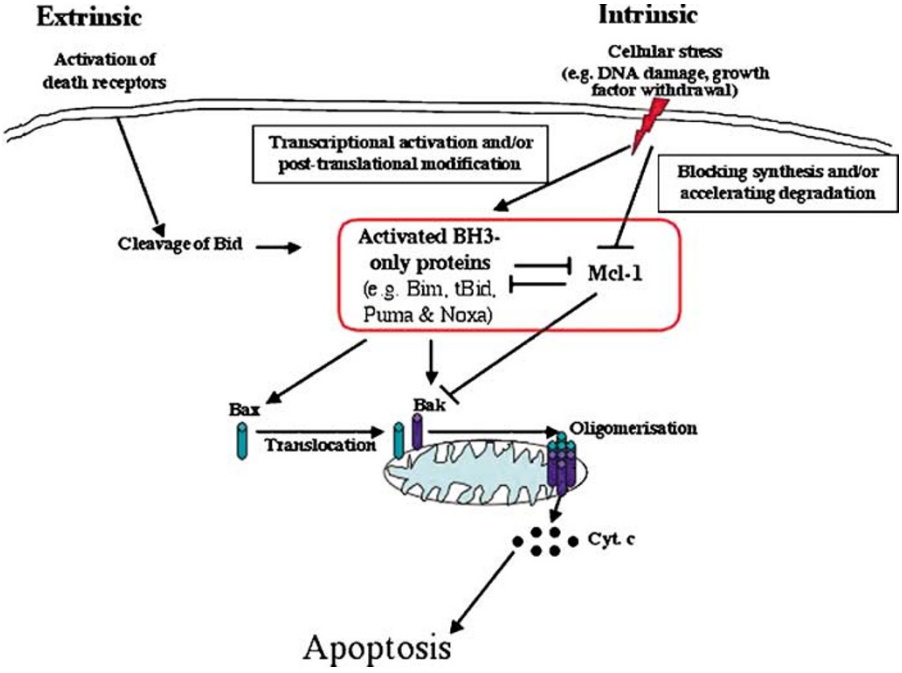

Figure 1 Mcl-1 actively counteracts BH3-only proteins in both intrinsic and extrinsic signalling pathways of apoptosis. Mcl-1 functions as an early critical negative regulator of apoptosis by directly interacting with BH3-only proteins including Bim, tBid, Noxa and Puma, and inhibiting their induction of cytochrome *c* release and activation of the mitochondrial apoptotic pathway (see text for details). In addition, Mcl-1 is also able to interact with Bak and to suppress its apoptotic activity. Mcl-1 thus functions at multiple sites in the apoptotic pathways, therefore playing a more active role in protecting cells from apoptosis than initially thought. It should be emphasised that, although BH3-only proteins require Bax and/or Bak to deliver their apoptotic functions, it still remains uncertain whether they activate Bax or Bak through direct protein-protein interaction

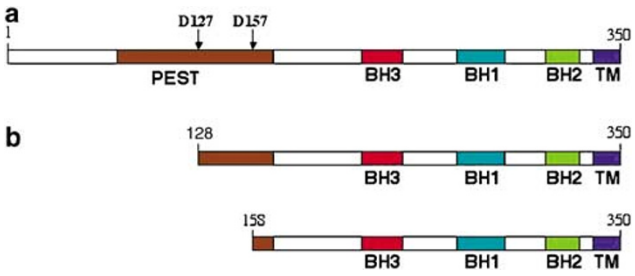

Figure 2 The functional domains of the human Mcl-1 protein. The BH (Bcl-2 homology), PEST and TM (transmembrane) domains of Mcl-1 are shown, along with the two aspartate residues at position 127 and 157 within the PEST domain identified as the caspase cleavage sites (**a**). For more detailed information on the structure of Mcl-1 see Day *et al.*[80] The Mcl-1 cleavage products are depicted in (**b**)

by N-terminal sequencing of the cleaved fragments of recombinant Mcl-1 protein following incubation with caspase-3.[78] The cleaved fragments resemble Bax-like protein containing the BH1, BH2 and BH3 domains (Figure 2b), it is therefore thought that they may be converted into proapoptotic molecules, as is the case with Bcl-2 and Bcl-x_L. The results from the functional studies are contradictory. Some suggest that the cleaved fragment(s) of Mcl-1 lose antiapoptotic potential and become proapoptotic,[48,78] whereas others show that the fragments alone do not exhibit any apoptotic activity.[59,79] We think that cleavage of Mcl-1 may be a common proteolytic feature of apoptosis following caspase activation. Its cleavage may disrupt the protective function of Mcl-1 ensuring the smooth progress of the cell death programme. Interestingly, this cleavage does not appear to interfere with the binding of Mcl-1 to the BH3-only proteins. When compared to full-length Mcl-1, cleaved forms of Mcl-1 lose the ability to protect against cell death induced by overexpression of Bim or tBid in HeLa cells although they are still able to interact with Bim[79] and tBid.[48] This indicates that the N-terminal domain of Mcl-1 is required for the inhibition of apoptosis, a conclusion compatible with the early reports that N-terminal BH4 domain of Bcl-2 and Bcl-x_L is essential for the antiapoptotic function. Regarding the hierarchy of apoptotic signalling, Mcl-1 likely functions as an apical regulator involved in cellular decision-making of life or death in response to an apoptotic stimulus, and acts before the activation of mitochondrial apoptotic pathway. Cleavage of Mcl-1 is a downstream event after activation of caspases, which may facilitate the complete demise of a cell that is beyond the commitment point to apoptosis.

Concluding Remarks

Accumulating evidence suggests that the antiapoptotic Bcl-2 family protein Mcl-1 plays a more active role in protecting cells from apoptosis than initially appreciated. Mcl-1 functions as an early critical negative regulator of apoptosis by directly interacting with BH3-only proteins including Bim, tBid, Noxa and Puma, and inhibiting their induction of cytochrome *c* release and activation of the mitochondrial apoptotic pathway. However, there are still many questions unanswered. For example, how specific is the interaction between Mcl-1 and BH3-only proteins? Does regulation of BH3-only proteins by Mcl-1 occur in a cell type-specific, stimulus-dependent manner or as a common mechanism? Mcl-1 is a potential target for therapeutic intervention in cancer as overexpression of Mcl-1 has been implicated in the pathogenesis of many forms of human leukaemia. However, as Mcl-1 is also essential for the maintenance of homeostasis of the haematopoietic systems, molecular targeting of Mcl-1 in tumour cells could render the normal haematopoietic cells more sensitive to apoptosis. Further studies identifying the molecular mechanisms that regulate Mcl-1 in physiological and pathological conditions may therefore help develop novel strategies that are applicable to cancer therapies while not disrupting normal haematopoiesis.

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