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Role of Bcl-2 family members on apoptosis: what we have learned from knock-out mice

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1. ABSTRACT

B-cell lymphoma-2 (Bcl-2) family members have been demonstrated to play a crucial role in the regulation of apoptosis as mediators in between the apical stimuli sensing steps and the executory mechanisms of apoptosis. Deregulation of their role may subvert the homeostasis of a given tissue and collaborate in the genesis of a myriad of diseases characterised by exacerbated or insufficient apoptosis, including diseases such as neurodegenerative diseases or cancer. Structural studies have defined homology regions shared by the members of the family that are responsible of the network of interactions established amongst the members of the family. These proteins usually form heterodimers between the so called antiapoptotic multidomain members and the proapoptotic BH3-only proteins. As a consequence, mitochondrial apoptogenic proteins are released to the cytoplasm and the apoptotic signal proceeds towards the final, execution phase of the apoptotic process. The high complexity of the family (more than 20 members have been isolated) makes the study of individual proteins difficult. Genetic approaches have revealed a high degree of redundancy in the family. Only a few proteins belonging to the antiapoptotic group have been proven to be essential for correct embryonic development. Genetic inactivation in mice shows a dramatic phenotype characterised by massive cell death in multiple tissues during embryogenesis, which leads from very early up to perinatal death. This genetic evidence proves the importance of the members of the family for the regulation of apoptosis in order to achieve the proper development and homeostasis of tissues and organs.

2. INTRODUCTION

The *bcl-2* gene was cloned in 1986 as the gene affected by the translocation t(14;18) present in about 85% of follicular lymphomas (1,2). As a result of this translocation, the *bcl-2* gene at chromosome segment 18q21 is juxtaposed with the Ig heavy chain locus at 14q32, resulting in deregulated expression of Bcl-2. The significance of Bcl-2 overexpression for the development of neoplasia was assessed by the generation of transgenic mice that carry the t(14;18) rearranged fragment in their germ line (3). Transgenic mice show indolent follicular hyperplasia of small non-cycling B cells that progresses towards diffuse large-cell lymphomas in old mice (4,5). Soon afterwards it was established that the oncogenic properties of the Bcl-2 protein were consequence of a mechanism completely different to the rest of the known oncogenes. Countless studies have established the anti-apoptotic effect of Bcl-2 in cell lines following the initial observation that Bcl-2 overexpression could block cell death in an IL-2 dependent cell line upon withdrawal of the cytokine (6).

3. THE BCL-2 FAMILY MEMBERS

The cloning in 1993 of the Bcl-2 homologous proteins Bax (Bcl-2-associated X) (7) and Bcl-x (8), demonstrated the existence of a family of Bcl-2-like apoptosis regulators. In addition to that, the study of Bax demonstrated that the expression of Bcl-2-like proteins did not always result in protection against apoptosis. Instead, the Bax-like proteins promoted cell death and, hence, Bax

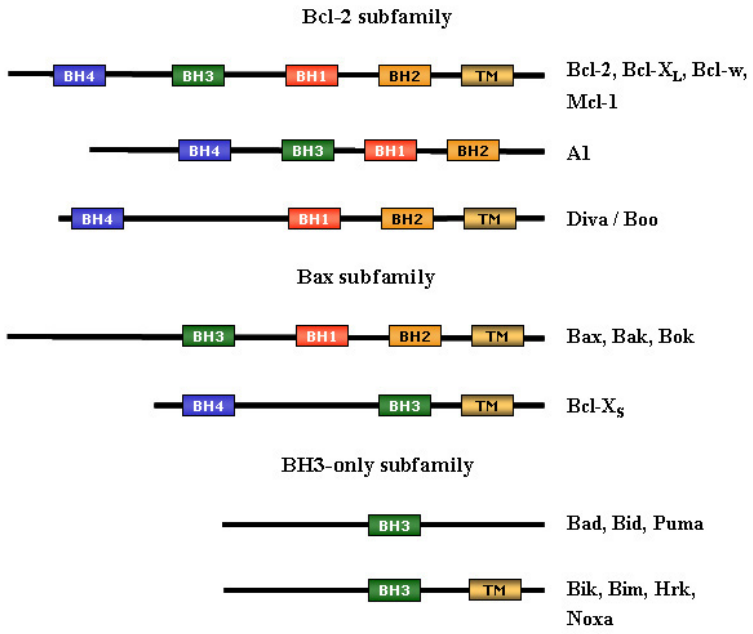

Figure 1. The Bcl-2 family members. According to their pro- or anti-apoptotic properties, the Bcl-2 protein family is subdivided in: Bcl-2 subfamily (anti-apoptotic proteins), all containing multiple BH domains; Bax subfamily (multidomain containing, pro-apoptotic proteins) and BH3-only subfamily (pro-apoptotic members containing only a single BH3 domain).

became the founder member of the proapoptotic subfamily of Bcl-2 family members. The cloning of the genes coding for the Bcl-2 family proteins has yielded up to 22 relatives, all of which share with it at least one conserved Bcl-2 homology (BH) domain. The members of the Bcl-2 and Bax subfamilies have sequences that are similar to those in Bcl-2, especially in the BH1, BH2 and BH3 regions (and are hence called multidomain members), but other members of the pro-apoptotic subfamily are homologous only in the short BH3 motif, an interaction domain that is both necessary and sufficient for their killing action (9). Figure 1 shows some of the best characterised Bcl-2 family members, classified in anti-death proteins (Bcl-2 subfamily, multidomain members) and pro-death proteins (Bax subfamily, multidomain proteins and BH3-only subfamily).

4. BIOCHEMICAL FUNCTION OF THE BCL-2 FAMILY MEMBERS

The biochemical function of the Bcl-2 family members has long been elusive. The elucidation of the three-dimensional structure of Bcl-X_L in solution uncovered its similarity with the membrane translocation domains of certain bacterial toxins that open pores in the cell membranes (10). This fact lead to the demonstration that Bcl-2 family members are able to form ion channels in synthetic membranes upon oligomerisation. These channels, however, would not be wide enough to allow the release of mitochondrial proteins such as Cytochrome c, mediators of the apoptotic signal, so they are most likely coupled to other proteins of the outer mitochondrial membrane. Even today, the details of the mechanism are not known, but it is generally accepted that Bcl-2 proapoptotic family members are able to regulate the release of the apoptogenic proteins from the mitochondrial intermembrane space (11). Hence, the proapoptotic members of the family would promote release of mitochondrial mediators (mainly Cytochrome c) while antiapoptotic proteins would prevent it.

5. THE BCL-2 FAMILY WORLD OF PROTEIN INTERACTIONS

The regulation of the biochemical action of the Bcl-2 related proteins is thought to be thanks to their general property of being able to form homo- and hetero-dimers. However, both the nature of the homo- and hetero-dimers that are formed in the cell and how this process is regulated are still being elucidated. It is considered that the hydrophobic side of the BH3 α-helix binds to the hydrophobic groove formed by the BH1, BH2 and BH3 domains of pro-survival members (12). In relation to this, several models explaining how BH3-only proteins work have been proposed. This section outlines the main traits of mice deficient in the best characterised Bcl-2 family members and discusses the recently proposed “hierarchical model” (13,14) in the light of the findings deduced from their phenotypes.

5.1. The classical or direct model

This model (Figure 2A), which is based on the direct interactions between anti and pro-apoptotic Bcl-2 family members that have been detected experimentally, proposes that the BH3-only proteins are activated in response to death-inducing stimuli, and function to neutralize pro-survival Bcl-2 proteins according to their relative affinity and free the Bax-like proteins to execute cell death (15,16).

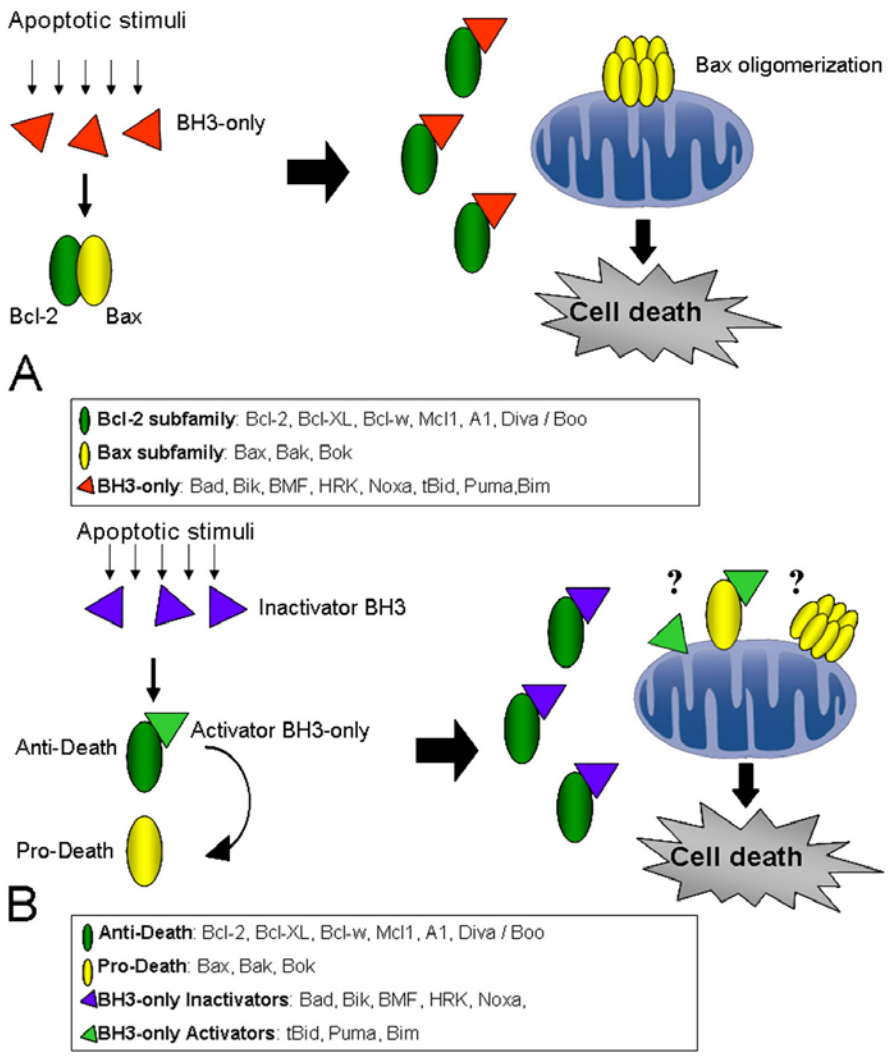

Figure 2. Two models to explain the functions of the Bcl-2 family network. (A) The classical or direct model. Bax and Bak proapoptotic members are constitutively inhibited by the Bcl-2 like proteins (Bcl-2, Bcl-XL, Bcl-w, A1, Diva, Mcl-1, red triangles). Upon arrival of the apoptotic signal, BH3-only proteins are activated and bind Bcl-2 like proteins releasing Bax and Bak. Bax and Bak can homo- and heterodimerize in the mitochondrial outer membrane, triggering the release of proapoptotic factors. (B) The hierarchy or indirect model. Anti-death proteins directly inhibit Activator BH3-only proteins. Upon arrival of the apoptotic signal, Inactivator BH3-only proteins (purple triangles) bind to Anti-death proteins, then releasing Activator BH3-only proteins. These either directly activate Pro-death proteins or cooperate with them to release proapoptotic factors from the mitochondria.

5.2.The hierarchy or indirect model

According to this model, BH3-only proteins can be divided in two subgroups, according to a hierarchy of interactions with different Bcl-2 family members each acting at a different step of the death pathway (Figure 2B). Similarly to the classical model, one subgroup of BH3-only proteins (BH3-only Inactivators), upon the arrival of an apoptotic stimulus binds directly the anti-death family members, leading to the release of their pro-death binding partners, the second group of BH3-only proteins (BH3-only Activators). These proteins (tBid: truncated BH3-interacting domain death agonist, Bim: Bcl-2-interacting mediator of cell death, and Puma: p53 upregulated modulator of apoptosis) can either lead directly to the release of mitochondrial apoptogenic proteins on their own or, alternatively, promote the activation of the pro-death members of the family by oligomerisation (Bax) (13,14) or displacement from binding to other proteins such as VDAC2 in the case of Bcl-2 homologous antagonist/killer (Bak) (17). However, recent biochemical and genetic evidences have challenged the role of the BH3-only Activator proteins in this model, showing that BH3-only proteins induce apoptosis indirectly by neutralising the relevant pro-survival proteins and allowing the activation of Bax and Bak to proceed (18) (reviewed in 19). Finally, and as a consequence of the release of apoptogenic mitochondrial proteins such as Cytochrome c, apoptosis inducing factor (AIF) or endonuclease G (Endo G), the

apoptotic signal proceeds through the assembly of the apoptosome and the activation of the executory Caspases.

6. THE KNOCKOUTS OF THE BCL-2 FAMILY MEMBERS

bcl-2 was the first member of the family to be studied by gene inactivation in mice (20). The complex and severe phenotype of *bcl-2* and *bcl-x* deficient mice suggested the generalisation that Bcl-2 family proteins are crucial cell death regulators that were essential for correct embryonic development (21). However, nowadays that most of the members of the family have been inactivated in mice, the conclusion is that, with the exception of myeloid cell leukemia factor 1 (Mcl-1), the phenotype of the rest of the knockout (KO) mice is mild, indicating a high degree of redundancy in the family.

In Table 1 are summarised the more relevant features of the phenotype of the single KOs of the Bcl-2 family members. Two main conclusions can be drawn: first, that only the single KOs of the antiapoptotic members of the family show a severe phenotype, while the rest of the KOs are viable, with phenotypes ranging from mild to non detectable. The second conclusion is that the more apparent phenotypes appear specially in two systems: the nervous and the haematopoietic systems. The reason for this could be either that the apoptotic processes regulated by the Bcl-2 family members are especially important for the development and physiology of these systems or, alternatively and especially in the cases where the phenotype of the KO mice is not lethal, because these systems have been traditionally better characterised due perhaps to their accessibility and availability of reagents for their study.

In Table 2 is summarised the available information about the double KOs described so far. The double KO of the proapoptotic multidomain members Bax/Bak has proven that they are redundant for a number of processes. The study of the double KO has revealed defective apoptosis in multiple tissues additionally to the phenotype observed in the Bax single KO mice (22). In the case of the Bak deficient mice, no apparent phenotype has been described (23). This particular case suggests that Bax and Bak belong to the same complementation group and constitute a subgroup inside the proapoptotic family members.

In the case of Bax and Bim double KO mice, the only change observed is a slight exacerbation of the single KO phenotypes, indicating that they work in related pathways but do not belong to the same complementation group. The attempts to rescue the phenotype of the Bcl-2 deficient mice by crosses with Bax, Bim or Bcl-2-interacting killer (Bik) KO mice have shown that only in the case of Bim KOs lead to amelioration of the Bcl-2 KO phenotype. In the rest of the cases, the Bcl-2 KO phenotype is dominant, indicating again that Bcl-2, Bax and Bik work in related but different pathways. Similarly, the Bcl-x KO is not rescued by crossing with the Bax KO, indicating that they work in different pathways.

Finally, to circumvent the redundancy between the BH3-only proteins, the cross between Bik and Bim KO mice has been generated (24). The only additional phenotype observed in the double Bik/Bim KO is the appearance of sterility. This lack of compound phenotype suggests that the subfamily may be very redundant and triple KOs may be necessary to uncover the functions of the members. Interestingly, Bim and Puma double KO mice show additional phenotypes not present in the single KOs, indicating functional compensation (25). However, Bim/BH3-interacting domain death agonist (Bid) double KO mice resemble single KO mice (18), again suggesting redundancy also inside this subgroup of BH3 only proteins.

7. THE HIERARCHY MODEL AT THE LIGHT OF GENETICS

The recently proposed hierarchy model (13,14), establishes functional groups inside the Bcl-2 family members. Should this model be true, the functional groups would show either functional compensation amongst themselves or be sufficient to play the assigned role inside the model.

Starting from the more homogeneous group, the anti-death members, this group includes the only genes of the family of which a KO is lethal, indicating that their function is necessary and sufficient and, with the exception of A1, Bcl-w and Diva/Boo, may form a homogeneous group. Perhaps A1, Bcl-w and Diva/Boo show functional compensation, especially Bcl-w and Diva/Boo that show a broader tissue expression pattern than A1 (data from http://www.proteome.com). Generation of double KO mice would help to clarify this point.

The main difference between the direct and indirect models is the subdivision of the BH3-only proteins into Activators and Inactivators. Hence, genetic proof of this division would greatly contribute to support the direct model. The Activator BH3-only proteins tBid, Puma and Bim are the subgroup of BH3-only proteins whose single KO mice show a more severe phenotype, specially the Bim KO mice. Compound inactivation of Bim and Puma uncovers new deffects respect to single KO mice (25), indicating functional redundancy and hence suggesting that they belong to the same complementation group. However, Bim/Bid double KO mice do not show additional phenotypes respect to single KO mice (18), again suggesting either a higher degree of redundancy than initially expected in this group of BH3-only proteins or that the protein groups defined by the hierarchy model needs to be further refined. The KOs of the rest of the BH3-only proteins (Inactivator BH3-only proteins), have very mild or non apparent phenotypes. The fact that all the BH3-only proteins show a broad expression pattern (data from http://www.proteome.com) that indicates coexpression of many of these proteins in a given tissue, leads us to speculate that this group of proteins is characterised by a high degree of redundancy. This is the group that includes the highest number of members and its positioning in the model is consistent with the broad variety of proapoptotic stimuli that a cell can receive and with the variety of

Table 1. Phenotype of the Bcl-2 family member knockout mice

		Molecule	Phenotype				References
			Viability	Nervous system	Haematopoietic system	Other	
Multidomain proteins	Anti-death	Bcl-2	Viable although 50% die 1-6 weeks after birth.		Increased sensitivity to apoptotic stimuli. Rapid loss of immune cells after birth.	Smaller in size (reduced long bone growth due to osteoblast dysfunction). Polycistic renal disease. Gray hair and hipopigmentation (due to melanocyte loss).	20, 31, 32, 33
		Bcl-X_L	Lethal at E13.	Extensive apoptosis in postmitotic immature neurons that lead to neuronal degeneration.	3-fold increased apoptosis in liver haematopoietic precursors. Decreased survival of immature lymphocytes and erythrocytes (using conditional knockout).		21,34,35
		Bcl-w	Viable			Male sterility and testicular degeneration.	36,37
		A1	Viable		Increased neutrophyl apoptosis.	Hair loss at face and head by 8-12 weeks of age.	38
		Mcl-1	Periimplantation lethality.		Profound reduction of B and T lymphocytes. Block of lymphocyte differentiation (using conditional knockout).	Periimplantation lethality is due to a trophoectoderm defect.	39,40
		Diva / Boo	Viable			No apparent phenotype.	41
	Pro-death	Bak	Viable			No apparent phenotype.	23
		Bax	Viable	Reduced developmental death of Purkinje and retinal precursors. Neuronal protection against injury.	Hyperplasic thymocytes and B-cells.	Increases in some cell types: granulosa cells, certain neurons and lymphocytes. Atretic ovaries with excess granulosa cells. Infertile males due to disordered seminiferous tubules with accumulation of premeiotic germ cells.	22
BH3-only	Inactivator BH3-only	Bad	Viable		5-fold increase in haematopoietic malignancy principally due to diffuse large B-cell lymphoma.	Male are fertile, although they have defects in testes due to prominent multinucleated giant cells in their seminiferous tubules. Cells are not sensitized to apoptotic stimuli when growth factors are withdrawed.	42
		Bik	Viable			No apparent phenotype.	43
		Hrk	Viable	Delayed neuronal apoptosis induced by NGF deprivation and axotomy.			44
		Noxa	Viable			MEFs have modest resistance to etoposide induced cell death.	45
	Activator BH3-only	Bid	Viable		Thymocytes are more resistant to death-receptor mediated apoptosis.	Resistance to Fas induced hepatocellular apoptosis.	46
		Bim	Viable, but born only 50% of the expected.		Blood leukocytes are increased. T-cell development is altered (abnormal CD4 / CD8 ratios). Lymphocytes are resistant to some apoptotic stimuli (cytokine deprivation, calcium ion flux, microtubule perturbation) but not to others. Plasma cells progressively increase by age up to 200-fold.	By age develop autoimmunity.	47
		Puma	Viable		Lymphocytes are resistant to genotoxic damage.		45

mechanisms of activation of the members. Hence, in a normal cell BH3-only proteins are kept in an inactive state by different mechanisms such as transcriptional control Puma and Noxa) (26), phosphorylation (Bad) (27), activation by Caspases (Bid) (28), association to microtubules (Bim and Bmf) (29,30), etc.

Table 2. Phenotype of the Bcl-2 family member double knockout mice

Molecules	Additional phenotype	Reference
Bax / Bak	• 90% die perinatally • Deafness and circling behaviour. • Retained interdigital webs and imperforate vaginas. • Increased number of hematopoietic progenitors, granulocytes and lymphocytes. • Thymocytes are resistant to DNA-damage induced cell death. • Persistence of a large of number of undifferentiated cells in the periventricular progenitor zones. • Progressive accumulation of mature B and T cells that lead to massive enlargement of the spleen and lymph nodes and infiltration of parenchymal organs.	23,48
Bax / Bim	• More severe defects in myeloid and B-lymphoid development. • Thymocytes are more resistant to apoptosis mediated by the intrinsic pathway.	49
Bak / Bim	• More severe defects in myeloid and B-lymphoid development. • Thymocytes are more resistant to apoptosis mediated by the intrinsic pathway.	49
Bik / Bim	• Sterility	24
Puma / Bim	• Exacerbation of haematopoietic defects present in Bim knockout. • Hyperplasia of lymphatic organs. • Increased spontaneous tumorogenesis.	25
Bim/Bid	• None	18
Bcl-2 / Bax	• Persistence of Bcl-2 KO phenotype	50
Bcl-2 / Bim	• Rescue of Bcl-2 KO phenotype	51
Bcl-2 / Bik	• Persistence of Bcl-2 KO phenotype	43
Bax / Bcl-x	• Persistence of major Bcl-x KO traits. (massive neuronal cell death is rescued).	52

Finally, the pro-death proteins have been proven to be highly complementary in the case of Bax and Bak (23), again showing that genetics support the functional group proposed by the model.

8. PERSPECTIVE

Since the isolation of Bcl-2, the founder member of the family, the importance of the Bcl-2 family proteins for apoptosis regulation has been extensively demonstrated by biochemical and genetic approaches. Their role as death regulators is important not only for the correct development of the organism, but also to preserve the homeostasis of the adult tissues. Perturbation of this equilibrium may contribute to diverse diseases, so it is crucial to keep functional the web of interactions among the different apoptosis regulators. Bcl-2 family members connect the mechanisms of sensing the different apoptotic stimuli and the detection of the damage infringed to the cell with the executory mechanisms of the process. Thus, their regulation is crucial to ensure the proper communication between the detection of the damage and the execution of the response. To ensure this, a big family of proteins with different regulation and properties has emerged. The functional redundancy between some of the members will ensure that the response to a myriad of different apoptotic stimuli takes place correctly. On the other hand, only a minority of the Bcl-2 family members have antiapoptotic properties, just enough to keep in check some of the proapoptotic members but they should not be expressed at levels high enough to block the cell death processes necessary for the correct tissue homeostasis. Future genetic work is likely to help to fully understand the complex surveillance machinery carried out by these proteins.

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Abbreviations: AIF: apoptosis inducing factor; Bad: Bcl-XL-associated death inducer; Bak: Bcl-2 homologous antagonist/killer; Bax: Bcl-2-associated X; Bcl-2: B-cell lymphoma-2; BH: Bcl-2 homology; Bik: Bcl-2-interacting killer; Bim: Bcl-2-interacting mediator of cell death, Bid: BH3-interacting domain death agonist, Endo G: endonuclease G (Endo G); KO: knockout; Mcl-1: myeloid

cell leukemia factor 1 (Mcl-1); Puma: p53 upregulated modulator of apoptosis; tBid: truncated Bid

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