ELSEVIER

Biochimica et Biophysica Acta 1271 (1995) 63–66

BBA Biochimica et Biophysica Acta

Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family

Stanley J. Korsmeyer *, Xiao-Ming Yin, Zoltán N. Oltvai, Deborah J. Veis-Novack, Gerald P. Linette

Division of Molecular Oncology, Departments of Medicine and Pathology, Howard Hughes Medical Institute, Washington University School of Medicine, 660 South Euclid, Box 8022, St. Louis, MO 63110, USA

Abstract

The maintenance of homeostasis in normal tissues reflects a balance between cell proliferation and cell death. Bcl-2 inaugurated a new category of oncogenes, regulators of cell death. The Bcl-2 gene was identified at the chromosomal breakpoint of t(14;18) bearing B cell lymphomas. Bcl-2 proved unique by blocking programmed cell death rather than promoting proliferation. In adults, Bcl-2 is topographically restricted to progenitor cells and longlived cells but is much more widespread in the developing embryo. Transgenic mice that overexpress Bcl-2 demonstrate extended cell survival, and progress to high grade lymphomas. Bcl-2 has been localized to mitochondria, endoplasmic reticulum and nuclear membranes, also the sites of reactive oxygen species generation. Bcl-2 does not appear to influence the generation of oxygen free radicals but does prevent oxidative damage to cellular constituents including lipid membranes. Bcl-2 deficient mice complete embryonic development but undergo fulminant lymphoid apoptosis of thymus and spleen. Moreover, they demonstrate two unexpected pathologies resulting from cell death, polycystic kidney disease and hair hypopigmentation. The latter is a potential oxidant injury from the melanin biosynthetic pathway. A family of Bcl-2 related genes is emerging that includes Bax, a conserved homolog that heterodimerizes in vivo with Bcl-2 and promotes cell death. The ratio of family members, such as Bcl-2/Bax, determines the survival or death of cells following an apoptotic stimulus.

Keywords: Oncogene; Bcl-2; Cell death; Reactive oxygen species

1. Bcl-2 initiates a new category of oncogenes: regulators of cell death

Malignancies usually possess aberrations in more than a single pathway [1]. Either increased proliferation or decreased death might result in an expansion of cell numbers (Fig. 1). To date, most of our knowledge concerning oncogenic events has concentrated upon mechanisms of increased growth and proliferation. Studies of Bcl-2 emphasize the existence of multiple pathways in the generation of neoplasia (Fig. 1). The increased cell number in neoplastic tissue can be viewed as a violation of normal homeostasis. The maintenance of homeostasis in normal tissue, in many respects, reflects a simple balanced equation of input (cellular proliferation and renewal) versus output (cell death). The maintenance of remarkably invariant cell numbers must reflect tightly regulated death pathways as well as controlled proliferation (Fig. 1).

Programmed cell death represents a cell autonomous suicide pathway that helps restrict cell numbers. The well-defined loss of specific cells is crucial during embryonic

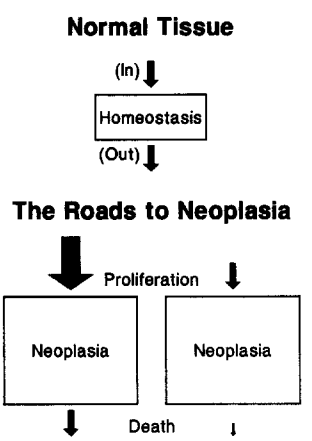

Fig. 1. Schematic representation of normal tissue homeostasis with balanced input and output reactions. Alternative roads to neoplasia are depicted as either increased proliferation (In) or decreased death (Out).

* Corresponding author. Fax: +1 (314) 4540486.

SSDI 0925-4439(95)00011-9

development [1]. In mature tissues, genetically programmed demise regulates the numbers of cells. A morphologically distinct and highly characteristic process entitled apoptosis accounts for these cell deaths [2]. Cells dying by apoptosis display marked plasma membrane blebbing, volume contraction, nuclear condensation, and the activation of an endonuclease that cleaves DNA into nucleosomal length fragments. Defining the precise genes and biochemical events that regulate this death program holds the promise for novel therapeutic approaches.

2. Bcl-2 represses programmed cell death

The t(14;18) (q32;q21) constitutes the most common chromosomal translocation in human lymphoid malignancies. Approximately 85% of follicular and 20% of diffuse B cell lymphomas possess this translocation [3,4]. Aberrant Ig heavy chain gene rearrangements on 14q32 proved to be the chromosomal breakpoint and delivered a candidate oncogene, Bcl-2, at 18q21 [5–7].

Whether the newly discovered genes found at chromosomal breakpoints could be shown to be transforming remained a major question. Bcl-2 conferred a death sparing effect to certain hematopoietic cell lines following growth factor withdrawal [8–10]. A stringent test of a gene's oncogenic capacity is to place it into the germline of mice to observe its effects during the development of an entire organism. Transgenic mice bearing a Bcl-2-Ig minigene initially displayed a polyclonal follicular lymphoproliferation that selectively expanded a small resting IgM/IgD B cell population. Cell cycle analysis confirmed that ~ 97% of the expanded B cells reside in Go/G1. These recirculating B cells accumulate because of an extended survival rather than increased proliferation [11,12]. Over time these transgenics progress from indolent follicular hyperplasia to diffuse large cell immunoblastic lymphoma [13]. A long latency period and progression from polyclonal hyperplasia to monoclonal high-grade malignancy is an indictment of secondary genetic abnormalities. Approximately half of the high grade tumors possess a c-myc translocation involving an IgH chain locus. These tumor cells have complemented an inherent survival advantage (Bcl-2) with a gene that promotes proliferation (myc). In addition to promoting cell cycle progression myc has been shown to promote apoptosis [14]. Thus, the overexpression of myc may specifically benefit from Bcl-2's ability to block apoptosis. Finally, the Bcl-2-Ig mice document the prospective importance of the t(14;18) in setting the stage for tumor progression and lymphomagenesis.

To assess the role of Bcl-2 in T cell development transgenic mice were generated in which Bcl-2 was redirected to the immature T cells in the cortex of the thymus [15]. A separate transgenic model also overexpressed Bcl-2 in the thymus and displayed similar effects [16]. The introduction of Bcl-2 into the normally vulnerable cortical thymocytes protected them from a wide variety of apoptotic stimuli including glucocorticoids, radiation, and anti-CD3 treatment. Moreover, Bcl-2 promoted T cell maturation. Bcl-2 enabled further T cell development obviating the need for several critical signals normally required for maturation [17]. This suggests that if cell death can be repressed inherent differentiation programs will manifest.

3. Bcl-2's localization: a clue to its function?

Bcl-2 has a COOH-terminal hydrophobic region that functions as a signal-anchor sequence responsible for the integral membrane position of the 25 kDa Bcl-2α product [18,19]. Bcl-2 has been localized most convincingly to mitochondria, endoplasmic reticulum and nuclear membrane [9,20,21]. Mitochondrial fractionation methods placed Bcl-2 predominantly in the inner membrane; whereas, immunoelectron microscopy and in vitro targeting showed more in the outer mitochondrial membrane. In vitro targeting studies indicate that while Bcl-2 can target microsomes, it selectively integrates into the outer membrane of mitochondria with its NH_2-terminus facing the cytosol [19]. COOH-terminus of Bcl-2 possesses a 19 amino acid hydrophobic tail bordered by positively charged amino acids that function as a signal/anchor sequence responsible for its integral membrane position. Of note the core of the Bcl-2 signal/anchor shares some homology with the corresponding region of the NH_2-terminal signal/anchor of the mitochondrial outer membrane protein in yeast, Mas70p. A Mas70p signal anchor peptide competed for insertion of Bcl-2 into the outer mitochondrial membrane but had no effect upon Bcl-2's association with endoplasmic reticulum. Indeed replacement of Bcl-2's signal/anchor with that of Mas70p resulted in a functional hybrid molecule that displayed a similar distribution to Bcl-2, still heterodimerized with Bax, and repressed cell death [22]. An anchor-minus form of Bcl-2, Bcl-2ΔC22, retained about half of its activity, but could still heterodimerize with membrane bound Bax [23]. Overall, the data favor a model in which Bcl-2 family members are targeted to strategic membrane locations within the cell, with the NH_2-terminus of the proteins available for cytosolic interactions.

4. Evidence for reactive oxygen species in cell death

Bcl-2's ability to block γirradiation-induced cell death is notable in that ionizing radiation amongst its effects produces hydroxyl radicals ($OH^{\cdot}$) by radiolytic attack on H_2O. The intracellular sites of oxygen free radical generation include mitochondria, endoplasmic reticulum and perhaps nuclear membranes; the sites where Bcl-2 has also been localized. Many of the effects of oxygen free radicals including DNA strand breaks and membrane blebbing

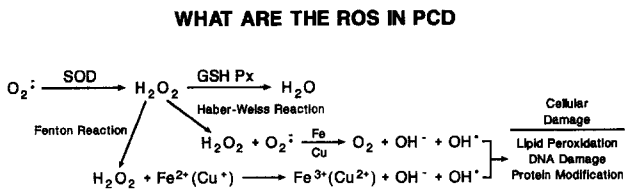

Fig. 2. Schematic representation of some major pathways of oxygen free radical generation and detoxification within cells that may be operative in cell death.

match the hallmark features of apoptosis, prompting an examination of Bcl-2's ability to counter other oxidative cell deaths. Bcl-2 protected cells from the lethal effects of H_2O_2 or *t*-butyl hydroperoxide in a dose-dependent manner [23,24]. At low concentrations these deaths resemble apoptosis. Bcl-2 also interfered with cell death induced by menadione, a quinone compound which undergoes redox cycles intracellularly to generate superoxide, O_2^-. Importantly Bcl-2 could not prevent the oxidative burst generated by menadione. Moreover, oximetry studies in two separate model systems of apoptosis revealed no evidence for any burst in the production of reactive oxygen species. Moreover, overexpression of Bcl-2 did not alter the efficiency of normal electron transport nor the production of peroxides. Consistent with this, rho° cells have been shown to undergo apoptosis which is also blocked by bcl-2 [21]. However, Bcl-2 completely suppressed damage to vital structures including the peroxidation of lipid membranes [23]. Consistent with this finding, overexpression of glutathione peroxidase (GSHPx), a known inhibitor of lipid peroxidation, repressed apoptosis. Which reactive oxygen species (ROS) might be important in cell death (Fig. 2)? Mutations within Cu/ZnSOD in familial cases of amyotrophic lateral sclerosis argue for an oxygen-radical mediated loss of motor neurons in that disease [25]. Data from apoptosis systems implicate peroxides as an important ROS perhaps reflecting the diffusibility of H_2O_2 or its proclivity for conversion to the most highly reactive $OH^{\cdot}$ radical (Fig. 2). Bcl-2 appears to block damage between peroxide generation and lipid membrane peroxidation. Whether this effect is direct or is several steps removed remains an important question. Bcl-2 has also been shown to inhibit necrotic forms of cell death. As H_2O_2 or menadione doses are increased the deaths resemble necrosis, and Bcl-2 displays an ability to retard these deaths within the lower range of oxidative stress. This also includes depletion of glutathione from neuronal cell lines [24]. If ROS prove to mediate demise in both apoptosis and necrosis it may be they represent a continuum of cell destruction rather than distinct pathways.

5. Bcl-2/bax: a cell autonomous rheostat controls cell death

Bcl-2 is a member of a multicomponent complex that includes a 21 kDa partner, Bax (Bcl-2 associated X protein) [26]. Of note Bax shares homology with Bcl-2 principally clustered within 2 highly conserved regions, Bcl-2 homology 1 and 2 (BH1 and BH2) domains (Fig. 3). It was possible that Bax worked in concert with Bcl-2 to repress death or that this association represented 'hand to hand' combat in which Bax promoted death while Bcl-2 opposed it. The latter proved to be the case in that excess Bax countered Bcl-2 and accelerated apoptotic cell death, but only following a death signal. Of note, the Bcl-2/Bax association exists in cells prior to a death stimulus. When Bcl-2 is in excess Bcl-2 homodimers dominate and cells are protected. When Bax is in excess Bax homodimers dominate and cells are susceptible to apoptosis (Fig. 4) [26]. A number of biologic systems indicate that cells vary during development in their inherent sensitivity or resis-

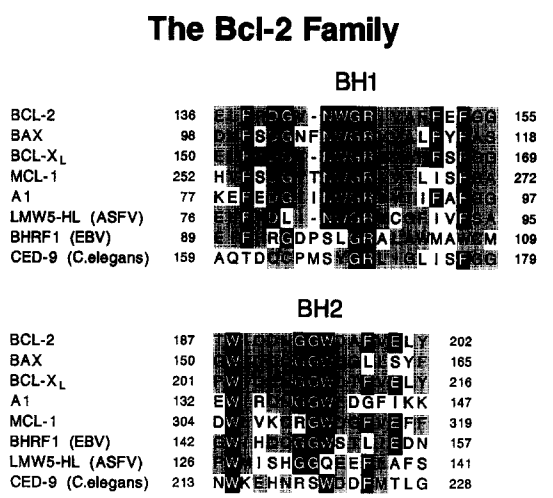

Fig. 3. Bcl-2 gene family. An expanding family of proteins homologous to Bcl-2 are most highly conserved within Bcl-2 homology 1 and 2 (BH1 and BH2) domains. Identical amino acids in black background and conserved positions in gray background.

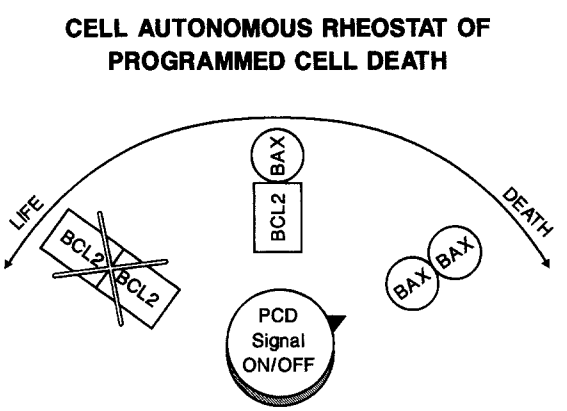

Fig. 4. Cell autonomous rheostat regulates cell death. Bcl-2 and Bax form homodimers and heterodimers. Following a signal for programmed cell death (PCD) cells die if Bax is in excess, but live if Bcl-2 predominates. Mutagenesis studies suggest that Bcl-2 homodimers may not be the active moiety.

tance to a given death stimulus. The ratio of Bcl-2/Bax represents one cell autonomous rheostat that pre-determines a cell's life or death response to an apoptotic stimulus.

6. Bh1 and bh2 are novel motifs which regulate dimerization

An emerging family of Bcl-2 related proteins are principally defined by the presence of conserved BH1 and BH2 domains (Fig. 3). The fact that Bax heterodimerizes with Bcl-2 and counters Bcl-2 suggested that these domains would participate in function or protein interaction. Site specific mutagenesis of Bcl-2 established BH1 and BH2 as novel dimerization motifs [27]. Single amino acid substitutions within BH1 or BH2 were identified that completely abrogated Bcl-2's death repressor activity. Of interest, those mutations that affected Bcl-2's function also disrupted its heterodimerization with Bax, yet still permitted Bcl-2 homodimerization. These data favor a model in which Bcl-2 must bind Bax to exert its activity, and suggest that Bcl-2/Bax heterodimers or Bax/Bax homodimers may prove to be the active pair in regulating death (Fig. 4).

7. Loss of function: bcl-2 deficient mice display fulminant lymphoid apoptosis

Within the embryo, Bcl-2 is expressed much more widely than in adult tissues. This raised questions as to whether this central repressor of cell death would be required for the successful development of multiple lineages. Bcl-2 deficient mice were created by gene targeting in embryonic stem (ES) cells [28]. Bcl-2 $-/-$ mice complete embryonic development and appear normal for the first week. The spectrum of hematopoiesis including B and T cell differentiation is initially normal. Thus, Bcl-2 is not absolutely required for the development of those lineages.

The development of most organs were histologically normal in Bcl-2 $-/-$ mice. However, after 1 week they display growth retardation, small external ears, immature facial features and early mortality. Bcl-2 $-/-$ mice display markedly hypoplastic renal development and progress to severe polycystic kidney disease (PKD). In particular Bcl-2 $-/-$ mice demonstrate fulminant metanephric apoptosis at embryonic day 12. This is especially prominent within the mesenchyme at the time of its induction by ureteric bud [29].

Hair growth in the mouse is cyclical. Bcl-2 $-/-$ mice have initially normal hair pigmentation. However, with the second hair follicle cycle Bcl-2 $-/-$ mice turn gray. A key intermediate in melanin synthesis, DOPA-quinone, is an extremely reactive compound that undergoes cyclization and oxidative polymerization to form the insoluble heteropolymers of eumelanin. In this process the production of light or dark melanin is regulated by cellular redox potential. Thus, in the absence of Bcl-2 melanin pathway derived free radicals could impact melanocyte survival in Bcl-2 $-/-$ animals.

Overtime Bcl-2 $-/-$ mice demonstrate fulminant apoptosis of the thymus and spleen and mice show nearly a complete loss of lymphocytes. Bcl-2 may have its most dramatic role in maintaining homeostasis in adult tissues. Of importance, Bcl-2 $-/-$ thymocytes require an apoptotic stimulus to manifest their predisposition to massive cell death. This is reminiscent of studies of overexpressed Bax. In many respects Bcl-2 deficient cells represent a rheostat reset to excess Bax/Bax (Fig. 4). The capacity of most organs to successfully complete development in Bcl-2 deficient mice is remarkable. This may reflect an expanding family of Bcl-2 related molecules, Bcl-x [30], Mcl-1 [31], A1 [32] (Fig. 3) that may provide redundancy in death repressor activity during development. Consequently strategies which alter ratios of Bcl-2 to Bax or disrupt Bcl-2/Bax heterodimers might be tolerated in many cell types yet prove an effective means to promote cell death in others.

References

[1] Glucksman, A. (1951) Biol. Rev. 26, 59.
[2] Kerr, J.F.R. et al. (1972) Br. J. Cancer 26, 239–257.
[3] Fukuhara, S. et al. (1979) Cancer Res. 39, 3119–3128.
[4] Yunis, J.J. et al. (1987) N. Eng. J. Med. 316, 79–84.
[5] Tsujimoto, Y. et al. (1985) Science 229, 1390–1393.
[6] Bakhshi, A. et al. (1985) Cell 41, 899–906.
[7] Cleary, M.L., Sklar, J. (1985) Proc. Natl. Acad. Sci. 82, 7439–7443.
[8] Vaux, D.L. et al. (1988) Nature 335, 440–442.
[9] Hockenbery, D. et al. (1990) Nature 348, 334–336.
[10] Nunez, G. et al. (1990) J. Immunol. 144 3602–3610.
[11] McDonnell, T.J. et al. (1989) Cell, 57, 79–88.
[12] McDonnell, T.J. et al. (1990) Mol. Cell Biol. 10, 1901–1907.
[13] McDonnell, T.J., Korsmeyer, S.J. (1991) Nature 349, 254–257.
[14] Evan, G.I. et al. (1992) Cell 69, 119–128.
[15] Sentman, C.L. et al. (1991) Cell 67, 879–888.
[16] Strasser, A. et al. (1991) Cell 67, 889–899.
[17] Linette, G.P. et al. (1994) Immunity 1, 197–205.
[18] Chen-Levy, Z. and Cleary, M.L. (1990) J. Biol. Chem. 265, 4929–4933.
[19] Nguyen, M. et al. (1993) J. Biol. Chem. 268, 25265–25268.
[20] Monaghan, P. et al. (1992) J. Histochem. Cytochem. 40, 1819–1825.
[21] Jacobson, M.D. et al. (1993) Nature 361, 365–369.
[22] Nguyen. M. et al. (1994) J. Biol. Chem. 269, 16521–16524.
[23] Hockenbery, D.M. et al. (1993) Cell 75, 241–251.
[24] Kane. D. et al. (1993) Science 262, 1274–1277.
[25] Rosen, D.R. et al. (1993) Nature 362, 59–62.
[26] Oltvai, Z. et al. (1993) Cell 74, 609–619.
[27] Yin, X.-M. et al. (1994) Nature 369, 321–323.
[28] Veis, D.J. et al. (1993) Cell 75, 229–240.
[29] Sorenson, C.M. et al. (1994) Am. J. Physiol. In Press.
[30] Boise, L.H. et al. (1993) Cell 74, 597–608.
[31] Kozopas, K.M. et al. (1993) Proc. Natl. Acad. Sci. USA 90, 3516–3520.
[32] Lin. E.Y. et al. (1993) J. Immunol. 151, 1979–1988.