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Bcl-2 family proteins as regulators of oxidative stress

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Abstract

The Bcl-2 family of proteins includes pro- and anti-apoptotic factors acting at mitochondrial and microsomal membranes. An impressive body of published studies, using genetic and physical reconstitution experiments in model organisms and cell lines, supports a view of Bcl-2 proteins as the critical arbiters of apoptotic cell death decisions in most circumstances (excepting CD95 death receptor signaling in Type I cells). Evasion of apoptosis is one of the hallmarks of cancer¹ (Hanahan and Weinberg), relevant to tumorigenesis as well as resistance to cytotoxic drugs, and deregulation of Bcl-2 proteins is observed in many cancers^{2,3} (Manion 2003, Olejniczak 2007). The rekindled interest in aerobic glycolysis as a cancer trait raises interesting questions as to how metabolic changes in cancer cells are integrated with other essential alterations in cancer, *e.g.* promotion of angiogenesis and unbridled growth signals. Apoptosis induced by multiple different signals involves loss of mitochondrial homeostasis, in particular, outer mitochondrial membrane integrity, releasing cytochrome c and other proteins from the intermembrane space. This integrative process, controlled by Bcl-2 family proteins, is also influenced by the metabolic state of the cell. In this review, we consider the role of reactive oxygen species, a metabolic by-product, in the mitochondrial pathway of apoptosis, and the relationships between Bcl-2 functions and oxidative stress.

Keywords

Apoptosis; mitochondria; reactive oxygen species

The extended Bcl-2 family

The discovery of BCL2 in 1985 as the oncogene harbored in t(14;18) translocations pathognomonic of follicular B-cell lymphomas was defining in several aspects. BCL2 became the first gene identified as an apoptosis regulator, soon bolstered by the reporting of

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ced-9, the homologous inhibitor of programmed cell death in *C. elegans*, and helped establish apoptosis as a specific pathway of cellular demise [4,5]. Prior to BCL2, oncogenes were recognized as promoting cell proliferation and transformation. As BCL2 lacked these effects, cooperating effects of oncogenes could be rationally understood in terms of complementing functions [6]. And finally, the localization of Bcl-2 protein to mitochondria provided an initial inkling of the importance of this organelle in apoptosis [7].

More than half of the Bcl-2 protein family promote apoptosis, in opposition to Bcl-2, including the second family member identified, BAX. All anti-apoptotic members and 3 pro-apoptotic proteins (Bax, Bak and Bok) contain 3 or 4 conserved domains (BH1–BH4) and a C-terminal transmembrane segment [8]. Three-dimensional protein structures of these proteins show globular proteins with similar all-alpha helical folds, without a clear-cut distinction at a structural level between those with pro- or anti-apoptotic functions [9]. The largest class of Bcl-2 proteins have a single conserved domain, the so-called BH3-only proteins. Although the Bcl-2 fold can be retained in this class, many of these proteins appear unstructured in solution [10]. Genomic sequencing efforts have revealed Bcl-2-related genes in all metazoan phyla examined to date.

Bcl-2 locus of action

Bcl-2 family proteins are found at mitochondrial and endoplasmic reticulum sites, as soluble proteins in cytosolic fractions, or bound to cytoskeletal elements [11]. The C-terminal tail of Bcl-2 functions as a signal anchor sequence for mitochondrial import, where it interacts with the import receptor Tom20 and the FKBP38 immunophilin, or delivery to the endoplasmic reticulum [12–15]. Several proteins, including Bcl-xL, Bax, and multiple BH3-only proteins, exhibit facultative targeting, with redistribution from cytosol-to-mitochondria in response to apoptotic stimuli [16]. In the case of multi-domain proteins, the C-terminal transmembrane domain is folded into a hydrophobic groove on the protein surface, requiring displacement by a BH3-only protein or conformational change affecting an N-terminal mitochondrial addressing signal [16–18]. BH3-only proteins expressed in healthy cells are sequestered by cytosolic proteins (Bad), attached to cytoskeletal elements (Bim), or exist as soluble, globular proteins (Bid).

The *sine qua non* of Bcl-2 survival function is mitochondrial retention of intermembrane space proteins including cytochrome c. Mislocalized cytochrome c acts as a cofactor for assembly of apoptosome complexes activating caspase-9. Release of other mitochondrial proteins competitively displace caspases-3, 7 and 9 from XIAP (SMAC and HtrA2/Omi), and induce caspase-independent chromatin condensation and DNA fragmentation (AIF, endonuclease G). The pro-apoptotic Bax and Bak proteins are leading candidates as apoptotic protein pores as a result of membrane embedding and oligomerization [19].

The terminal conformational changes leading to pore assembly are promoted by interactions with BH3-only proteins and membrane lipids [20]. The anti-apoptotic functions of Bcl-2 proteins can be explained in this model by inhibitory associations with the pore-forming Bax and Bak proteins or activating BH3-only proteins [21]. In some cases, Bcl-2 inhibits changes

in N-terminal conformation of cytosolic Bax and Bax translocation to mitochondrial sites, suggesting a mechanism of action that does not require physical association [22,23].

Competing models for cytochrome c release during apoptosis involve osmotic swelling of the mitochondrial matrix with subsequent rupture of the less-expandable outer membrane. Mitochondria isolated from growth factor-deprived cells are insensitive to respiratory control by external ADP and accumulate creatine phosphate in the intermembrane space, consistent with closure of the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM) [24]. Inner mitochondrial membrane hyperpolarization resulting from ATP/ADP exchange leads to matrix swelling. Bcl-xL expression sustains OMM permeability under these conditions, and increases the VDAC gating potential in reconstituted preparations [25]. Similar changes were not observed with Fas- or TNF-induced apoptosis, suggesting alternative Bcl-xL-inhibited mechanisms for cytochrome c release [24]. Bcl-2 also inhibits opening of mitochondrial permeability transition pores, another mechanism of matrix swelling, induced by diverse agents (atractyloside, uncoupling agents, alkylperoxides), although the relevance of mitochondrial permeability transition for apoptosis and Bcl-2 has been called into question [26–28].

Extra-mitochondrial functions of Bcl-2 can also sustain mitochondrial integrity. Activation of ER-localized Bax or Bak results in release of Ca^{2+} stores, which can promote cytochrome c release through ER-mitochondrial crosstalk. Bcl-2/Bcl-xL expression affects Ca^{2+} transport, independently of Bax/Bak, through direct interactions with the inositol 1,4,5-triphosphate receptor and sarco/ER Ca^{2+} -ATPase [29–31].

Oxidative stress in apoptosis

Apoptotic cell death can be triggered by a very long list of environmental conditions, pharmacologic agents, imbalanced signal transduction pathways, and minor damages to various cellular constituents. A majority of studies in the literature have shown that reducing oxidative stress prevents apoptosis, including instances without obvious pro-oxidant triggers. Examples include glucocorticoid-induced death of T-lymphocytes [32–35], hyper- and hypo-thermia [36–40], growth factor or serum deprivation [41–46], TNF, FasL, and TRAIL-engaged death receptor signaling [47–61], viral infection [62,63], c-myc- and p53-induced apoptosis [64–68] and staurosporine-induced cell death [69–72].

Counter-arguments against a role for oxidative stress in apoptosis have been raised, including the ability for apoptosis to proceed normally in hypoxic environments. Normal apoptotic responses to staurosporine, anti-Fas antibody, and IL-3 deprivation occur in cells grown in very low oxygen environments (< 8 ppm) to eliminate reactive oxygen species (ROS) formation [73,74]. Other studies have demonstrated that ROS are still produced by submitochondrial particles at low oxygen tensions, generated by near-complete oxidation of succinate and to a lesser extent, NADH [75]. Hypoxia paradoxically increases superoxide production, primarily at complex III of the electron transport chain [76]. This appears to be due to the maintenance of normoxic levels of flux through the electron transport chain (ETC) at very low oxygen tensions (half-maximal O_2 consumption < 45 ppm at low [ATP]/[ADP]•[Pi] ratios) [77]. Cytochrome c oxidase (complex IV) activity is reduced in hypoxia

with a compensatory shift toward a reduced state of cytochrome c and more proximal electron carriers [78,79], leading to superoxide (O_2^-) production at complex III [80,81]. The ability to induce apoptosis under stringently anoxic conditions suggests that ROS are dispensable for apoptosis under these conditions [82], although equilibration with liquid media and oxygen absorption by plasticware may complicate interpretation of hypoxia experiments [83,84]. Surprisingly, ρ^0 cells lacking mitochondrial DNA and a functional electron transport chain, are resistant to anoxia-induced apoptosis. This raises the interesting possibility that mammalian cells are capable of anaerobic mitochondrial metabolism for redox balance or ATP generation during anoxia, e.g. using nitrite as a terminal electron acceptor, as occurs in plants [85,86]

Although outnumbered by positive studies, a number of studies have failed to demonstrate inhibition of apoptosis using anti-oxidants [87,88]. One caveat in interpreting these studies is the potential for anti-oxidant compounds to exhibit pro-oxidant effects due to formation of chain initiating radicals or hormesis phenomena [89–91]. The tendency to restore cellular redox equilibrium may explain paradoxical inhibition of apoptosis by pro-oxidant interventions [92].

Location and targets of oxidative stress

There are multiple sources of reactive oxygen species in cells, representing most subcellular compartments. Mitochondria have generally been considered a principal source, although estimates range from 2–5% to 0.15% of total O_2 consumption [93,94]. Superoxide generation occurs by single electron leakage to O_2 at complex I, complex III and possibly the electron transfer flavoprotein-quinone oxidoreductase complex for fatty acid oxidation. Mitochondrial superoxide production is influenced by substrate fuel and respiratory state, and the site of production can be mapped by determining the effect of inhibiting electron transfer at different sites in the ETC. Superoxide generated at complex I is released at the matrix side of the inner mitochondrial membrane, and fed by forward flow of electrons from NADH or reverse flow from the complex II substrate succinate [94]. Rotenone inhibition of complex I increases superoxide produced by NADH-generating substrates, but inhibits superoxide supported by reverse electron flow [95]. Reduction of O_2 at complex III is principally via the ubisemiquinone radical at center *o*, with release into the intermembrane space. Antimycin increases superoxide production at complex III by preventing oxidation/release of ubisemiquinone at center *o*, while myxothiazol inhibits superoxide by binding to center *o* and preventing initial oxidation of ubiquinol to ubisemiquinone.

If mitochondria-produced ROS play a critical role in apoptosis, it would be useful to know if there is a predominant site of O_2^- generation, either as a result of a common upstream stimulus or a required downstream target [96]. There is more support for complex I as an origin of ROS in the apoptosis literature, including TNF- or ceramide-induced and Parkinsonian models of apoptosis, with some indications that reverse electron flow may be involved [47, 97–99]. Mitochondrial complex I is inhibited by nitration and glutathionylation [100,101], as well as proteolytic inactivation by caspases and granzyme A [102,103].

Caspase-independent deaths with mitochondrial release of AIF and endonuclease G, but not cytochrome c, are usually associated with complex I-dependent superoxide production [104–108]. Whether this is related to the functional interactions between AIF, an NADH oxidase flavoprotein, and complex I (NADH dehydrogenase) activity, is unknown.

Several studies are consistent with complex III as a ROS source [109–112]. Cytochrome c, the electron acceptor reduced by complex III, also functions as a scavenger of superoxide released into the intermembrane space [113]. A significant fraction of cytochrome c, a basic protein, binds to the anionic phospholipid cardiolipin at the outer surface of the inner mitochondrial membrane, where it acquires peroxidase activity [114,115]. Cytochrome c peroxidase activity subtracts electrons from nearby unsaturated acyl chains, setting in motion progressive lipid peroxidation. Oxidation of inner mitochondrial membrane cardiolipin is an early apoptotic event required for subsequent cytochrome c release [116,117]. As lipid-soluble antioxidants can block cardiolipin oxidation, this could be a sufficient explanation for apoptosis inhibition by antioxidants. However, increased cytochrome c peroxidase activity in apoptosis is also likely to be driven by increased H₂O₂ substrate, which may be derived from matrix or intermembrane space superoxide due to the ability of H₂O₂ to cross membranes readily [96].

Other sites proposed for ROS generation during apoptosis are the plasma membrane and endoplasmic reticulum. Exposure of phosphatidylserine (PS) in the outer leaflet in the plasma membrane serves as a phagocyte recognition signal. Oxidation of PS acyl groups, as a result of the peroxidase activity of released cytochrome c, promotes externalization of PS [118]. Another ROS source, the NADPH oxidase gp91phox expressed in phagocytic cells, and homologous Nox and Duox catalytic subunits expressed in neurons, endothelial cells and other cell types, transfer cytoplasmic electrons across membranes to generate superoxide. In phagocytic cells, NADPH oxidase assembles with adaptor proteins on phagocytic vacuoles. ROS produced by NADPH oxidase have been implicated in apoptotic cell death in both phagocytic and non-phagocytic cells, usually based on inhibition of apoptosis using flavoenzyme inhibitors such as diphenylene iodonium [46, 119–122]. NADPH oxidases in non-phagocytic cells are thought to function in signaling pathways, and may also be recruited to endosomal vesicles in apoptotic cells upstream of Fas/CD95 activation [123]. There are also necrotic cell deaths linked to NADPH oxidase, and a potential role of NADPH oxidase in preventing caspase activation has been reported [124,125].

Unfolded proteins within the endoplasmic reticulum initiate stress response pathways to suppress general protein synthesis while inducing ER resident chaperones, ER membrane expansion and retrotranslocation of unfolded proteins for proteosomal degradation [126]. The unfolded protein response (UPR) also includes increased ROS generation in the ER, perhaps by an increased load on the oxidative protein folding pathway or increased GSSG/GSH ratio from reducing misplaced protein disulfides. Retrieval of oxidizable, misfolded protein substrates by ER-associated protein degradation can relieve oxidative stress within the ER [127,128]. Several ER-associated proteins with UPR functions have been shown to suppress ER-dependent ROS during apoptosis, including the Hsp70-related chaperone Grp78, TRAF2, and paraoxonase [129–131]. Endoplasmic reticulum-localized

cytochrome P450 systems are also a potential source of ROS [132–136]. Mitochondrial ROS production is often found to increase with ER stress [128]. Specific ER targets of ROS relevant to apoptosis are largely unknown, although the sarco/endoplasmic reticulum Ca-ATPase (SERCA) is regulated by NO-dependent S-glutathionylation and subject to non-reversible oxidation [137].

Oxidative stress and Bcl-2

Bcl-2 was initially reported to have anti-oxidant effects in 1993 [138, 139]. Apoptotic deaths induced by dexamethasone and growth factor deprivation exhibited progressive lipid peroxidation that was completely suppressed with Bcl-2 transfection. As addition of anti-oxidants also deterred apoptosis, oxidative stress appeared causal rather than secondary to apoptosis in these experiments. Unexpectedly, no increase in ROS accounting for the increased lipid peroxidation could be detected in these models using fluorescent reporters of O_2^- and H_2O_2 or measurement of cyanide-resistant respiration. Bcl-2 also rescued cells treated with menadione, without affecting cyanide-resistant O_2 consumption by bioreductive metabolism of this quinone. Similar findings were reported with *t*-BuOOH [140]. These results suggested that Bcl-2-expressing cells are intrinsically resistant to added oxidative stress, without directly lowering ROS levels. Conversely, apoptotic stimuli could increase oxidative damage, apparently due to endogenous (i.e. normal) production of ROS.

Cellular ROS levels can be decreased by Bcl-2. In cells depleted of glutathione, Bcl-2 expression reduced intracellular ROS levels, consistent with an effect on ROS clearance [139]. Lower ROS levels are also observed in Bcl-2-expressing cells treated with arsenic trioxide, an inhibitor of thioredoxin reductases [141]. Bcl-2 has been reported to increase cell levels or shift intracellular distribution of reduced glutathione [142–145] and a recent paper described direct binding of glutathione to Bcl-2 [146]. However, Bcl-2 does not appear to substitute for glutathione, as Bcl-2-mediated survival can be negated if reduced glutathione is sufficiently depleted in media lacking cysteine and methionine [147, 148].

Antioxidant effects of Bcl-2 should be evident in non-apoptotic contexts, for consideration as a primary or proximate function of Bcl-2. One example of this type is the observation that hyperglycemia-induced lipid peroxidation and advanced glycation end products (AGE) in endothelial cells are suppressed by Bcl-2 [149]. Unlike the classic antioxidants desferoxamine, α -tocopherol and dimethylsulfoxide, which inhibit ROS generation as well as lipid peroxidation/AGE formation, ROS levels were unaffected by Bcl-2 in these studies. Perhaps as a related phenomenon, Bcl-2 was shown to increase the activity of plasma membrane $Na^+/K^+-ATPase$, a redox-sensitive ion pump in unstimulated cells [150, 151]. The anti-oxidant phenotype with Bcl-2 expression in mammalian cells is also demonstrated by heterologous expression of Bcl-2 in *Saccharomyces cerevisiae*, an organism lacking Bcl-2 orthologs. Yeast transformed with expression plasmids for Bcl-2, Bcl-xL or the *C. elegans* ortholog CED-9 are resistant to oxidative stress, while SOD-deficient strains of the budding yeast, are partially rescued by Bcl-2 [139, 152].

Bcl-2^{-/-} mice have increased content of oxidized proteins in multiple tissues [153,154]. Retinal endothelial cells from these mice also exhibit *in vitro* defects in capillary

morphogenesis that can be restored by treatment with N-acetylcysteine [155]. Conversely, Bax expression in mammalian and heterologous models increases ROS generation and oxidative damage [156–162].

An opposite effect of Bcl-2 to increase cellular levels of ROS has been reported in HL-60 cells depleted of glutathione [163,164], bacteria [165], astrocytes [166], several cancer cell lines [167,168], and fibroblasts [169]. These observations have led to the proposal that Bcl-2 produces a mild increase in cellular oxidative stress, leading to increased anti-oxidant capacity within the cell and better ability to withstand additional oxidative stress. Such effects may also be related to expression level [170,171].

An interesting perspective on the relationship between Bcl-2, ROS and mitochondrial function was recently presented in relation to treatment with mitochondrial complex inhibitors [172]. CEM lymphoblastic leukemia cells and HeLa cells transfected with Bcl-2 were noted to have higher rates of respiration and cytochrome oxidase activity than control cells, along with higher mitochondrial production of O_2^- using the MitoSOX Red fluorescent reporter. Therefore, cellular ROS levels may normally increase with a shift toward mitochondrial ATP production. Treatment with rotenone or antimycin A (complex I and III inhibitors, respectively) increased mitochondrial O_2^- generation in control cells, but not Bcl-2-expressing cells. The flat O_2^- response in Bcl-2-expressing mitochondria was associated with depressed cytochrome oxidase activity, suggesting to the authors that Bcl-2 prevented excessive mitochondrial ROS production by reducing mitochondrial ETC activity.

As discussed above, mitochondrial effects of Bcl-2 are not restricted to limiting ROS production. Alterations in mitochondrial membrane potential (MMP) are commonly observed in apoptotic cells, most often membrane depolarization that can occur early or delayed relative to other apoptotic changes [173]. In contrast, several groups have described an early increase in MMP after apoptotic stimuli including camptothecin, staurosporine, Fas ligation, glucose and IL-3 withdrawal [174–180]. Notably, Vander Heiden *et al.* demonstrated that Bcl-xL prevents mitochondrial hyperpolarization in the setting of IL-3 withdrawal [175]. Apoptosis induced by growth factor deprivation is associated with decreased outer mitochondrial membrane permeability, resulting in impaired ATP/ADP exchange. In the presence of Bcl-xL, adenine nucleotide exchange is restored. Experiments with isolated mitochondria and synthetic lipid membranes indicate that Bcl-xL maintains an open conformation of the outer membrane voltage-dependent anion channel (VDAC).

Loss of outer mitochondrial membrane permeability in the presence of adequate substrates to reduce electron carriers in the respiratory chain, is expected to increase mitochondrial O_2^- generation. Ubisemiquinone radicals at center *o* in complex III are stabilized at the higher membrane potentials that are no longer used for ATP synthesis. The anti-oxidant effect of Bcl-2 might be explained as an indirect consequence of promoting adenine nucleotide exchange. However, VDAC closure is not observed with other apoptotic cues [24], including ischemia-reperfusion injury in which Bcl-2 appears to have the opposite effect of decreasing outer mitochondrial membrane permeability [181].

The availability of small molecule inhibitors for Bcl-2 and Bcl-xL affords a unique opportunity to observe the effect of acutely interrupting survival function on mitochondrial physiology [182]. By examining transfected cells overexpressing Bcl-2/Bcl-xL, responses due to inhibition of protein function can be isolated from those associated with removal of an essential survival factor. Treatment of a murine hepatocyte cell line overexpressing Bcl-xL with the Bcl-xL inhibitor, 2-methoxy antimycin A (2-MeAA), led to a progressive increase in mitochondrial membrane potential measured by TMRM fluorescence after 20–30 min. Image analysis of mitochondrial and cytosolic NAD(P)H using 2-photon excitation demonstrated a concurrent elevation in mitochondrial NAD(P)H. This response could be explained by inhibition of ATP/ADP exchange or increased substrate-driven NAD(P)H availability. In favor of the latter interpretation, fluorescence recovery after photobleaching (FRAP) studies demonstrate that the rate of NAD(P)H regeneration in mitochondria increases after 2-MeAA treatment and O₂ consumption rate is unchanged at the time of initial increase in mitochondrial NAD(P)H (unpublished data).

These results suggest that Bcl-xL may “unload” the mitochondrial respiratory chain, thereby lessening O₂[−] generation, or reductive stress within mitochondria. Bcl-xL-transfected TAMH cells are shifted toward glycolytic metabolism compared to control transfectants, with increased lactate production and reduced O₂ consumption. Similar phenotypes were observed in mice expressing a Bcl-xL transgene in pancreatic islet β-cells, resulting in reduced insulin responses to hyperglycemia, tet-inducible constructs in PC-12 cells, and HeLa cells [183–185]. Notably, this phenotype is consistent with the Warburg effect, suggesting that Bcl-2 survival factors contribute to this characteristic feature of cancer cell metabolism.

By acting as a “safety valve” for mitochondrial electron transport chain flux, Bcl-2 may also improve mitochondrial function by reducing chronic oxidative stress. In some cases, this could be manifest as an increase in mitochondrial respiration, as observed in some studies. Examples of this functionality include uncoupling proteins in mammalian species and alternative oxidases in plants and lower eukaryote species [186, 187].

Bcl-2 lacks sequence or structural features of known anti-oxidant or redox active proteins, suggesting that anti-oxidant effects are indirect. The most likely explanation is that Bcl-2 modulates mitochondrial bioenergetics, i.e. availability of substrate fuels, electron flow, inner mitochondrial membrane leakiness or coupling to adenine nucleotides in a direction that diminishes known mechanisms of O₂[−] generation within mitochondria. Although framing Bcl-2 function in this manner is less straightforward and more admitting of inconsistencies in the literature, final elucidation of Bcl-2’s connection to oxidative stress is likely to yield some novel insights for mitochondrial physiology.

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