BCL-2 FAMILY PROTEINS IN DEVELOPMENT AND TREATMENT OF MALIGNANT DISEASES

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Abstract

Proteins of Bcl-2 family are key regulators of mitochondrial pathway of apoptosis. Deregulation of apoptosis disrupts the complex and delicate balance between cell proliferation, survival and death and plays an important role in the development of malignant diseases. In addition to uncontrolled proliferation, alterations in apoptotic proteins are frequently associated with resistance of malignant cells to chemotherapy, leading to ineffective treatment with chemotherapy that primarily acts by apoptosis initiation. Despite the progress in combinatory and biologic therapy, response rates for treatment of different malignant diseases are not high enough. Therefore, new anti-cancer agents that selectively kill tumour cells and spare normal tissues are still urgently needed. Progress in biochemistry and cell biology leading to detailed dissection of cell signalling pathways allows development of new therapeutic strategies targeting different proteins involved in malignant transformation and uncontrolled proliferation of malignant cells. Emerging knowledge on molecular mechanisms of apoptosis deregulation in cancer development has revealed Bcl-2 family proteins as potential targets for drugs discovery. Structural analysis of these proteins together with studies of apoptosis mechanisms have outlined strategies for generation of new drugs, resulting in numerous novel chemical entities with mechanism-based activity. Many of the most logical targets for promoting apoptosis of malignant cells are technically challenging, involving often disruption of protein interactions or changes in gene expression, as opposed to traditional pharmaceutical approach that predominantly attacks enzymes. Understanding of the core components of the apoptotic machinery at the molecular and structural levels may lead to new era in cancer therapy where the intrinsic and acquired resistance of malignant cells to apoptosis can be pharmacologically reversed, reinstating natural pathways of cell suicide.

Key words: Bcl-2 family proteins, apoptosis, cancer therapy, chemoresistance

INTRODUCTION

Apoptosis is an evolutionarily conserved mechanism of cell death that is crucial for the development of multicellular organisms. Apoptosis is also critical for the maintenance of normal tissue homeostasis by the removal of damaged, unnecessary or potentially dangerous cells. Unlike necrosis, apoptosis is characterized by typical morphologic and biochemical features such as cell shrinkage, condensation of nuclear chromatin and cleavage of genomic DNA at internucleosomal sites, resulting in the generation of a characteristic pattern of DNA fragments [1]. Blebbing of the cell surface results in the release of membrane-bound apoptotic vesicles. Cells undergoing apoptosis are eliminated by macrophages or neighbouring cells without inflammation of surrounding tissue. The majority of molecular changes during apoptosis are caused by family of intracellular cysteine proteases, called caspases. Caspases synthesised as inactive zymogens are proteolytically processed to an active form following apoptotic stimulus [2]. Two groups of caspases are recognized according to their functions. Initiator caspases (caspases 2, 8, 9, 10) are capable of autocatalytic activation in response to different stimuli. Active initiator caspases are capable of proteolytic cleavage and consequent activation of effector caspases (caspases 3, 6, 7) that can cleave various cellular substrates such as cytoskeletal proteins or proteins involved in signal transduction. Cleavage of target proteins is associated with cell collapse and induction cell death [3]. In addition, proteolytic degradation of inhibitor of caspase activated DNase leads to intranucleosomal fragmentation of genomic DNA. There are two distinct pathways that lead

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to apoptotic cell death: (1) the extrinsic or receptor pathway; and (2) the intrinsic or mitochondrial pathway [1, 2].

The extrinsic pathway is initiated by the binding of extracellular ligands (e. g. members of tumour necrosis factor super family) to their receptors on plasma membrane, which induces formation of the death-inducing signalling complex (DISC) by binding to specific intracellular proteins characterised by presence of death domain [2]. DISC promotes activation of initiator caspase 8 and consequent activation of effector caspase 3. Degradation of substrate proteins by active caspase 3 culminates in apoptotic cell death.

The intrinsic pathway is usually initiated in response to intracellular stress, which include hypoxia, DNA damage, viral infection and activation of oncogenes. Proteins of Bcl-2 family are central regulators of mitochondrial pathway of apoptosis [4]. The *BCL-2* (B-cell lymphoma gene-2) gene was first cloned from the breakpoint of the t(14;18) translocation in B-cell follicular lymphoma [5]. Its discovery has led to identification of a whole family of proteins that control commitment to apoptosis via mitochondrial pathway [4]. To date, 25 members of the Bcl-2 family have been identified. In addition to cytoplasm, these proteins are located predominantly in the outer mitochondrial membrane [2]. Proteins of the Bcl-2 family are related to each other due to their four conserved Bcl-2 homology (BH) domains and can be classified into three subfamilies based on structural and functional features [4].

The anti-apoptotic subfamily contains the Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bfl1/A-1 and Bcl-B proteins, which suppress apoptosis and contain all four BH domains, designated 1-4. The 3-dimensional structures of anti-apoptotic Bcl-2 proteins reveal that each has a similar overall helical fold centered on a core hydrophobic helix with the BH1-3 domains arranged to expose a hydrophobic groove on the molecule. The integrity of this hydrophobic groove is required both for their pro-survival activity and for the binding of their pro-apoptotic partners.

Pro-apoptotic proteins, such as BAX, BAK, and BOK, contain BH 1-3 domains and are termed "multidomain proteins", whereas other pro-apoptotic proteins, such as BIM, BAD, BID, PUMA and Noxa contain only BH3 domain and are termed "BH3-only" proteins. The BH3 domain of pro-apoptotic proteins is the most important mediator of the interaction with anti-apoptotic proteins. In viable cells, the „multidomain“ pro-apoptotic proteins are present in the form of inactive monomers residing at the mitochondria (BAK), where are bound to anti-apoptotic proteins Bcl-xl or Mcl-1, or in the cytosol (BAX) [6]. In response to multiple types of stress, BAX translocates to mitochondria. Monomeric BAX and BAK undergo a conformational change leading to dimerisation and formation of oligomers during apoptosis. These changes occur at mitochondrial membranes and are promoted by local availability of selected BH3-only proteins (BIM, BID, and PUMA) [7]. Oligomerisation of BAX and BAK leads to pore formation in outer mitochondrial membrane followed by the release of cytochrome c and other pro-apoptotic proteins from mitochondria to cytosol, activation of initiator caspase 9 and consequent activation of caspase 3 and apoptosis initiation [4].

The „BH3-only“ proteins bind due to BH3 domain into the hydrophobic groove of anti-apoptotic proteins inhibiting their anti-apoptotic function and indirectly activate BAX and/or BAK. The balance between pro- and anti-apoptotic members of Bcl-2 family expressed in outer mitochondrial membrane determines apoptosis initiation or cell survival. Disruption of this complex equilibrium may result in overinduction of apoptosis or by contrast in regression of apoptosis [8]. In addition to the regulation of cell cycle and DNA repair, p53 is an important regulator of mitochondrial apoptosis at the level of Bcl-2 family proteins. It can initiate apoptosis by transcriptional activation of the expression of pro-apoptotic proteins (e.g. BAX, BAK, PUMA and Noxa) [9], as well as transcriptional repression of the expression of anti-apoptotic proteins of Bcl-2 family [10]. Transcription-independent pathway involves translocation of p53 to mitochondrial membrane and its direct binding to anti-apoptotic proteins (e.g. Bcl-xl); thereby inhibiting their anti-apoptotic function [11].

DEREGULATION OF BCL-2 FAMILY PROTEINS IN MALIGNANT CELLS

Deregulation of apoptosis plays an important role in the development of a variety of human pathologies, including autoimmune, neurodegenerative and malignant diseases [1]. The identification of the components of apoptotic pathways has enabled the detection of various biochemical defects present in malignant cells compared to their normal counterparts [12]. These defects contribute to the survival advantage of malignant clone over the normal cells and are also frequently associated with low response rate to standard chemotherapy and poor survival of patients [13]. Significant correlation between expression of some proteins of Bcl-2 family and sensitivity of cancer cell lines to more than 100 cytostatics was documented [14]. Dissection of molecular pathway of apoptosis has lead to the identification of many potential targets for the development of new drugs targeting anti-apoptotic molecules abnormally expressed or deregulated in malignant cells. Many of these drugs have potential to restore the sensitivity of malignant cells to apoptotic stimuli and some of them are under investigation at a clinical level [15]. Molecular mechanisms involved in deregulation of mitochondrial apoptosis in malignant cells include overexpression of anti-apoptotic proteins and down-regulation or loss of function of pro-apoptotic proteins.

OVEREXPRESSION OF ANTI-APOPTOTIC PROTEINS

The overexpression of proto-oncogene *BCL-2* was first identified in 70% of human follicular B-cell lymphomas due to t(14;18) chromosomal translocation [5]. The same translocation, however with less frequency (28%), was detected in diffuse large B-cell lymphomas

Table 1. Association of Bcl-2 Anti-apoptotic Family Members with Human Malignant Diseases

Protein	Diagnosis	Reference
Bcl-2	Neuroblastoma	24
	Small-cell lung carcinoma	25
	Acute and chronic lymphoid leukaemias	19, 20, 26
	Acute myeloid leukaemia	27
	Breast adenocarcinoma	28
Bcl-xl	Prostate adenocarcinoma	29
	Lung adenocarcinoma	30
	Hepatocellular carcinoma	31
	Glioma	32
	Colorectal adenocarcinoma	33
	Acute lymphoblastic leukaemia	34
	Renal carcinoma	35
Mcl-1	B-cell non-Hodgkin lymphomas	36
	Anaplastic large cell lymphoma	37
	Multiple myeloma	38
	Melanoma	39
Bcl-w	Gastric adenocarcinoma	40
	Colorectal adenocarcinoma	41

(DLBCL) [16]. This translocation places the *BCL-2* gene into juxtaposition with powerful enhancer elements associated with immunoglobulin heavy chain locus, which results in elevated levels of Bcl-2 mRNA and protein [17]. In addition to t(14;18) chromosomal translocation, t(2;18) and t(18;22) translocations that involve Bcl-2 and either *IG kappa* or *lambda* genes have been observed in some rare cases of B cell malignancies. The molecular analysis revealed that these translocations juxtaposed the *BCL-2* and *immunoglobulin light-chain (IGL)* genes [18] and is cause of Bcl-2 overexpression in approximately 10 % of cases of chronic lymphoblastic leukaemia (CLL) [19]. Bcl-2 was found to be overexpressed in all investigated cases of CLL [20]. Although hypomethylation of the *bcl-2* gene did not necessarily correlate with the relative levels of Bcl-2 protein present in the B-CLL cells [20], this epigenetic event represents additional mechanisms for regulating Bcl-2 expression in cancer cells. Increased expression of Bcl-2 protein was also described in 31% of DLBCL due to *BCL-2* gene amplification which was independent from t(14;18) chromosomal translocation [21]. Overexpression of the apoptotic protein Bcl-2 provides a block in apoptosis that can be frequently observed also in other types of cancers (Table 1). The up-regulation of Bcl-2 can be used as prognostic marker in some cancer types while it does not correlate with disease progression in other types [22]. In the most cases, overexpression of Bcl-2 is associated with resistance to chemotherapy. For example, high level of Bcl-2 in acute myeloid leukaemia (AML) is associated with poor remission outcome and patient survival [23].

Alternative splicing of *BCL-X* gene gives two variants, long variant Bcl-xl and short variant Bcl-xs that are translated to proteins with opposite functions. Bcl-xl is the most potent anti-apoptotic protein of Bcl-2 family that is often up-regulated in malignant cells due to replacement of wild type exon IA of *BCL-X* gene with exon IB. The promoter found upstream of exon IB is 300-fold more potent than "wild" type promoter [42]. Bcl-xl is also upregulated in many types of malignancies (Table 1). On the other hand, association of decreased level of mRNA of anti-apoptotic Bcl-xs with shorter disease free and overall survival indicates important association of *BCL-X* gene products with chemotherapy induced cell death [43].

In addition to Bcl-2 and Bcl-xl, increased expression of other anti-apoptotic proteins of Bcl-2 family (e. g. Mcl-1, and Bcl-w) occurs in significant subsets of common types of malignant diseases (Table 1) and is generally correlated with poor response. However, the exact cause of overexpression of these proteins is not yet fully elucidated.

DOWN-REGULATION OR LOSS OF FUNCTION OF PRO-APOPTOTIC PROTEINS

Down-regulation of BAX, the main pro-apoptotic pore forming protein of Bcl-2 family, is often observed in some solid tumours. In the most cases, the reduced level of BAX protein is accompanied by mutations in the *P53* gene. According to its function in cell death, *P53* gene is frequently mutated in solid tumours [44], leading to expression of inactive p53 protein which cannot activate transcription of *BAX* gene. Frameshift mutations of *BAX* gene have been detected in T cells of acute lymphoblastic leukaemia (ALL) [45] and endometrium [46]. In all cases of gastrointestinal cancers, there are two characteristic missense mutations of *BAX* gene in codon 169 (Thr → Ala or Thr → Met). These mutations are functional and therefore, they inhibit the pro-apoptotic activity of the protein and contribute to carcinogenesis [47]. Finally, Rampino et al. [48] found that more than 50% of human matrix metalloproteinase positive (MMP+) colon adenocarcinomas had frameshift mutations in the third exon, spanning codons 38 to 41. These results suggest that inactivating *BAX* gene mutations are selected during the progression of colorectal MMP+ tumours and that the wild type BAX protein plays a suppressor role in a p53-independent pathway for colorectal carcinogenesis.

Reduced levels of BAK, another pore forming protein, were detected in gastric tumours [49] and colorectal adenocarcinomas [33]. This down-regulation may be explained by the presence of missense mutations in the *BAK* gene [50]. Six missense mutations were found by analysis of the *BAK* gene in the carcinomas of uterine cervix, while no mutations were

detected in normal cervical tissues [51]. *BAK* mutations were observed more frequently in the advanced stage, at which mutated cancer tissues were resistant to radiotherapy [51].

THERAPY OF MALIGNANT DISEASES TARGETING THE BCL-2 FAMILY PROTEINS

The fact that high expression of anti-apoptotic proteins of Bcl-2 family may confer resistance to chemo- or radiotherapy initiated the effort in developing strategies that overcome the cytoprotective effect of these proteins. Less effort has been made in search of strategies restoring the activity of pro-apoptotic proteins. Attempts to overcome the cytoprotective effects of anti-apoptotic proteins include 3 strategies: (1) shutting off gene expression; (2) inducing mRNA degradation; and (3) direct attack of the proteins with small-molecule inhibitors [52].

Drugs regulating gene expression

Several types of drugs regulating the expression of anti-apoptotic Bcl-2 family genes were found. Some synthetic retinoids reduce levels of Bcl-2 or Bcl-xl mRNA in malignant cells (e. g. fenretinide) [52]. Fenretinide has shown promising anticancer activity in preclinical studies, but its limited oral bioavailability has hindered clinical assessment.

Also inhibitors of histone deacetylases (HDACs), chromatin-modifying enzymes, have the ability to reduce the expression of *BCL-2*, *BCL-X* or *MCL-1* at transcriptional level. For example, sodium butyrate, inhibitor of class I HDAC, induces apoptotic cell death accompanied by up-regulation of pro-apoptotic BAX and down-regulation of anti-apoptotic Bcl-2 and Bcl-xl [53]. Clinical trials of HDAC inhibitors are progressing, with hints of activity documented for lymphoma and some solid tumours [54]. The use of valproic acid that acts also as a class I HDAC inhibitor has shown promising results in patients with AML and myelodysplastic syndromes [55].

Drugs attacking mRNA

Antisense oligonucleotides are short single stranded synthetic deoxyribonucleotides that complement and bind specific coding regions on mRNA, forming DNA/RNA hybrids which are subsequently degraded. Thus, degradation of mRNA prevents translation of the targeted protein. This approach is highly specific since any DNA sequence longer than 17 nucleotides occurs only once within human genome. Preclinical studies have demonstrated that oblimersen sodium (Genasense; G3139), an 18-mer phosphorotioated oligodeoxynucleotide antisense designed to bind the first six codons of human Bcl-2 open reading frame, downregulates expression of its target [56]. The feasibility of this approach in vivo was investigated in several phase II/III trials of oblimersen alone or in combination with other therapy in both solid tumours and haematological malignancies [52]. In a recent phase III study, oblimersen combined with fludarabine/cyclophosphamide was shown to prolong survival of a subset of patients with refractory or relapsed CLL [57]. Oblimersen also holds promise for the treatment of metastatic melanoma [58].

Small-molecule inhibitors of the Bcl-2 family proteins

Intensive research using computational modeling has led to discovery of structures of new small molecules that could potentially promote death of cancer cells. Designed molecules mimic the function of BH3-only pro-apoptotic proteins, destroying cancer cells by inhibiting activity of anti-apoptotic proteins and specifically targeting pathways that allow survival of cancer cells [59]. The attempt to target the protein-protein interaction site between anti-apoptotic Bcl-2 proteins and BAX or BAK has resulted in the generation of ABT-737, which binds with high affinity ($K_i \leq 1$ nM) to Bcl-2, Bcl-xl and Bcl-w, but not to the less homologous proteins Bcl-B, Mcl-1 and A1 ($K_i > 1$ μ M) [60]. ABT-737 binds directly into the hydrophobic groove in the structure of anti-apoptotic proteins. Thus, ABT-737 inhibits anti-apoptotic Bcl-2 family proteins, but does not directly activate the pro-apoptotic proteins

BAX and BAK. ABT-737 is an effective single agent in a panel of different malignancies, especially in those that critically depend on anti-apoptotic Bcl-2 proteins for survival [60, 61]. Using of ABT-737 as a therapeutic agent is obstructed by its poor pharmaceutical properties. This compound is not orally bioavailable and has low solubility in water. The second-generation inhibitor ABT-263, that is orally bioavailable, is currently evaluated in phase I/II trials for lymphoma and CLL [62] as well as small cell lung carcinoma [63]. However, inhibition of Bcl-2, Bcl-xl and Bcl-w by ABT-263 has only moderate effects on survival of different types of malignant cells and additional inhibition of Mcl-1 is required to activate cell death [60, 62]. Therefore ABT-263 may have limited clinical potential for the treatment of glioma and other solid tumours [64]. However, ABT-263 and related compounds display synergism with chemo- and radiotherapy, significantly reducing the median effective concentration (EC_{50}) value for cytotoxicity of the chemotherapeutic in a variety of cancer cell lines [60].

Obatoclox (GX15-070) mesylate is a molecule that can also supplement the activity of ABT-263. Although obatoclox mesylate has a lower affinity than ABT-263 for Bcl-2, Bcl-xl and Bcl-w, it can successfully prevent Mcl-1/BAK binding [65]. Phase I studies in different malignancies demonstrated evidence for single-agent activity and was well tolerated. Clinical response to obatoclox mesylate was observed in patients with refractory leukaemia [66], with CLL [57] and lymphoma or advanced solid tumours [67]. Its safety profile makes it an attractive candidate for its combinatorial use with traditional chemotherapy [68]. Promising results were obtained during phase I trials using obatoclox mesylate in combination with topotecan in patients with relapsed small cell lung carcinoma [69].

In addition to synthetic compounds, natural substances are tested for treatment of different malignant diseases. Gossypol is a natural polyphenolic compound and BH3 mimetic derived from cottonseeds, which possesses pro-apoptotic effects in various *in vivo* and *in vitro* models [70]. Similar to obatoclox, gossypol acts as a pan-Bcl-2 inhibitor and can inactivate Bcl-2, Bcl-xl, Mcl-1 and Bcl-w. There are two enantiomers of gossypol, (+)-gossypol and (-)-gossypol. The latter being more potent tumour growth inhibitor, (-)-gossypol (AT-101), has shown single-agent activity in various types of malignant diseases. Although gastrointestinal toxicity is considerable dose limiting factor [52], its potential impact for cancer therapy is currently investigated in phase I/II clinical trials. As a single agent AT-101 is tested for treatment of prostate cancer [71] and gliomas [64]. AT-101 combined with topotecan at a reduced dose is currently investigated in phase I/II clinical trials for treatment of relapsed and refractory small cell lung carcinoma [72].

A combination of drugs targeting several anti-apoptotic proteins may be necessary to overcome resistance to apoptosis in some cancers. For such reason, recent research is directed not only at investigation of clinical effects of already discovered inhibitors, but also at identification of new small molecules that could make stable complexes simultaneously with Bcl-2, Bcl-xl and Mcl-1. A few small molecules were obtained by computer-based modification of known molecules. Usefulness of the new designed molecules for anticancer drug development is waiting for determination [59].

CONCLUSIONS

The development and progression of the malignant diseases invariably involve deregulation of apoptosis. Despite major efforts during the past 40 years, limited improvements in response rates and overall survival have been made. The current primary treatment for malignant diseases is chemotherapy inducing cell death by apoptosis mainly mediated through the mitochondrial pathway. The progress made in understanding of the central role of apoptosis deregulation in the development of malignant diseases and chemoresistance has launched the academic community, as well as the pharmaceutical and biotechnology industry into a search to identify new compounds for the treatment of malignant diseases. Targeted therapies that are designated to induce apoptosis in malignant cells are currently

the most promising anticancer strategies. These strategies are aimed at targeting and killing the malignant cells specifically with no or limited collateral damage to normal cells. Finally, further elucidation of the molecular apoptotic machinery as well as its defects in malignant diseases lays the basis for developing new drugs able to trigger apoptosis of malignant cells, and therefore to improve survival of cancer patients.

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