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Commentary

Role of Bcl-2 and its post-transcriptional modification in response to antitumor therapy

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Abstract

Bcl-2 blocks or delays apoptosis in many cell systems. The protein exerts its antiapoptotic effect mainly in the membrane of mitochondria. Indeed, emerging evidence supports that the mitochondrion plays an important role in the cell death pathway, integrating different pro- and antiapoptotic stimuli. Since deregulation of the expression of Bcl-2 occurs in a variety of human tumors, modulation of its function is regarded as an exploitable manipulation for pharmacological intervention in antitumor chemotherapy. Phosphorylation of Bcl-2 has been implicated as an important regulatory mechanism of its function and is a common event in response to antimitotic drugs. Recently, a similar post-transcriptional modification was observed in response to DNA-damaging agents in some tumor systems, but this is not a general finding in response to genotoxic drugs. Current investigations indicate that different signaling pathways may be involved in Bcl-2 phosphorylation, likely dependent on the kinases activated by the various stress stimuli. A better understanding of the molecular mechanisms by which Bcl-2 regulates apoptosis could provide insights for a rational design of approaches to enhance the susceptibility to drug-induced cell death.

Keywords: Bcl-2; Phosphorylation; Drug response

1. Introduction

Apoptosis has received much attention, because this form of cell death seems to be the predominant cellular response to effective antitumor treatment [1,2]. Thus, susceptibility to drug-induced apoptosis is recognized as a critical determinant of tumor cell sensitivity to antitumor agents. Although drug resistance of tumor cells may involve multiple factors, there is evidence to support the hypothesis that cancer cells can exhibit resistance to cytotoxic agents as a result of alterations of apoptosis-related proteins [1,3]. The ability of the cell to undergo apoptosis and the threshold at which a specific cell injury triggers the process critically influence drug efficacy and the therapeutic index of drug treatment.

At least three functionally distinct phases of apoptotic cell death have been identified [4]. The biochemical pathway of the initiation phase depends on the nature of the lethal stimulus (receptor- or stress-mediated induction). The downstream events (effector and degradation phases) involve regulatory gene products (including activators and repressors) that modulate the apoptotic response. Among the known genes that regulate apoptosis induced by a wide variety of cytotoxic stimuli, the Bcl-2 gene family plays an important role [5]. The gene products of the Bcl-2 family include a number of proteins, characterized by amino acid sequence homology [4], that have been implicated in regulating the effector stage of the apoptotic pathway. Like Bcl-2, some members act by inhibiting apoptotic cell death. An opposite (proapoptotic) function has been described for other Bcl-2-related proteins (e.g. Bax and Bad). Bcl-2 was originally found in human B-cell lymphomas in which it becomes deregulated as a result of chromosomal translocation [6].

Since high levels of Bcl-2 expression have been detected in a variety of tumor types, additional mechanisms, besides chromosomal translocations, appear to be involved in the deregulation of Bcl-2 expression. In particular, a negative regulation of Bcl-2 transcription by the *p53* tumor suppressor gene has been reported [6]. Since loss of p53 function following mutation is a very common alteration in human tumors, the finding of Bcl-2 up-regulation in a large variety of tumors is not surprising. On the basis of its overexpression and antiapoptotic function, Bcl-2 has been implicated in drug resistance [3,6]. Protection by Bcl-2 and related antiapoptotic proteins against cell death induced by a vari-

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ety of antitumor agents has been proposed as a novel mechanism of multidrug resistance [6]. In spite of the complex and controversial mechanism of apoptosis regulation by the Bcl-2 family protein [5], an obvious possible therapeutic intervention to overcome resistance to apoptosis is the manipulation of the Bcl-2-mediated control system, since the function of Bcl-2-related proteins resides at a critical step upstream of irreversible cellular damage (i.e. caspase activation) [4].

2. Bcl-2 and drug resistance

Based on the well-established role of Bcl-2 as a critical regulator of the cell death process, overexpression of this protein is expected to confer a relative resistance to the cytotoxic effects of antitumor therapies. However, despite the functional importance of Bcl-2 and related proteins in apoptosis control, the relationship between Bcl-2 expression and drug resistance of tumor cells remains controversial. Much of the confusion may be related to the complex regulation of the apoptotic response involving the Bcl-2 pathway. Overexpression of Bcl-2 and Bcl-x_L following transfection has been shown to confer resistance in leukemia cells to a large variety of cytotoxic agents including DNA-damaging agents [6,7]. Conversely, down-regulation of the expression of Bcl-2 using antisense oligonucleotides has been proposed as a novel strategy for reversal of resistance of leukemia and lymphoma cells that overexpress Bcl-2 [8–10].

In contrast to tumor cells of haematopoietic origin, the relevance of Bcl-2 in cells of solid tumors is still unclear. No correlation was found between Bcl-2 levels and chemoresistance of ovarian and lung carcinoma cell lines [11–13]. Expression of exogenous Bcl-2 in the ovarian carcinoma cell line A2780 resulted in a low level of resistance to cisplatin and in delayed drug-induced apoptosis [14]. A delay in drug-induced apoptosis was not associated with an increase in clonogenic survival of Bcl-2 transfected HeLa cells [15]. A similar observation has been reported for Bcl-2-overexpressing Jurkat cells [16]. There is no consistent pattern of Bcl-2 expression across various breast or ovarian carcinoma cell models with variable sensitivity to DNA-damaging agents. The breast carcinoma cell line MCF-7 ADR, selected for resistance to doxorubicin, is characterized by down-regulation of Bcl-2 [17,18]. Conversely, the human breast carcinoma MX-1, characterized by overexpression of Bcl-2, is hypersensitive to cisplatin and to a variety of cytotoxic agents [19–21]. In human ovarian carcinoma cell lines, overexpression of Bcl-2 (either naturally occurring or produced by gene transfection) confers a trend toward sensitivity but not resistance to cisplatin [13]. Similarly, Bcl-2 transfection does not afford protection of the ovarian carcinoma cells A2780 against cytotoxicity of doxorubicin [22].

The resistance of Bcl-2 overexpressing ovarian carcinoma cells could be related to additional factors that may contribute to the resistant phenotype. For example, the observation that the *p53* gene is a regulator of Bcl-2 and Bax expression [6] provides a plausible explanation for the overexpression of Bcl-2 in cells resistant to DNA-damaging agents [23]. Indeed, mutations of *p53*, a common alteration in ovarian carcinoma, could result in a relative resistance to DNA-damaging agents as a consequence of reduced susceptibility to p53-dependent apoptosis. In this biological context, the partial suppression of apoptosis in response to genotoxic lesions is not simply related to the mere overexpression of Bcl-2. In addition, the loss of transcriptional suppression of other drug resistance-related factors (e.g. MDR and MRP) as a consequence of inactivation of *p53* [24,25] could explain the co-expression of various resistance mechanisms, including overexpression of Bcl-2 [26].

3. Regulation of Bcl-2 function

Since the cellular response following a cytotoxic injury is dependent on a complex decision that involves coordinated signal transduction pathways affecting the cell death machinery, it is evident that the alteration of a single regulatory component, like Bcl-2, is not sufficient to determine the final outcome. Even among leukemia cells, there is evidence that Bcl-2 expression may not be effective in suppressing apoptosis in all circumstances [27]. Lack of cell protection by elevated levels of Bcl-2 could reflect: (a) the presence of high levels of antagonistic proteins (e.g. Bax); (b) post-translational modifications of the Bcl-2 protein that modulate or inactivate its function; and (c) activation of cell death signals that bypass the Bcl-2-mediated control of apoptosis [4].

Proapoptotic and antiapoptotic members of the Bcl-2 family are localized in the outer mitochondrial membrane, but Bcl-2 has also been detected in the nuclear membrane and in endoplasmic reticulum [4,5]. The regulatory function of Bcl-2 has been ascribed to: (a) its ability to interact with other proteins that participate in cell-death regulation; and (b) channel activity that directly or indirectly influences mitochondrial permeability and prevents the release of cytochrome *c* from the mitochondria and caspase activation [3]. Thus, the mitochondrion has been recognized as the primary site for the regulatory functions of Bcl-2 [28].

Several studies have provided evidence that a number of biochemical events in the apoptotic pathway converge on mitochondria [5,28]. Several mitochondrial dysfunctions, including changes of membrane potential, generation of reactive oxygen species, and release of calcium and cytochrome *c*, precede the nuclear features of apoptosis. The Bcl-2 antiapoptotic protein acts by stabilizing the mitochondrial membranes, in contrast to a destabilizing function of Bax. Additional evidence for the specific function of Bcl-2 is the finding that overexpression of Bcl-2 prevents the biochemical perturbations of mitochondria and apoptosis

induced by lonidamine, a selective inhibitor of energy metabolism and mitochondrial functions [17,18,29]. Relevant to this point is the observation that the potentiation of the antitumor effects of cisplatin by lonidamine is associated with enhanced phosphorylation of Bcl-2 [19]. Bcl-2 is known to be phosphorylated in response to different cell death stimuli [30]. Post-translational phosphorylation is a common regulatory mechanism in signal transduction pathways [31]. However, the functional significance of post-translational modifications of Bcl-2 in regulating apoptosis remains controversial. Bcl-2 phosphorylation has been related to inactivation or activation of the antiapoptotic function [32–37]. Since these studies were performed in cells of different tumor types, including leukemia and prostate carcinoma, it remains to be defined whether the different biological context or the nature of the cytotoxic stress influences the pattern of cellular response. The interpretation of the functional role of Bcl-2 phosphorylation may be confounded by the possibility that Bcl-2 may be phosphorylated by different kinases involved in pathways not necessarily related to cell death.

The inability of phosphorylated Bcl-2 to heterodimerize with the Bax protein is consistent with the view that phosphorylation would activate Bcl-2 [36]. Bcl-2 phosphorylation, extensively studied in cells treated with antimicrotubule agents, is considered a specific hallmark of cytotoxicity of the drugs [37,38]. Indeed, among a variety of cytotoxic agents, only drugs that affect microtubule integrity were reported to be able to induce phosphorylation of Bcl-2, whereas the effect was not observed after treatment with DNA-damaging agents [37,39]. Such behaviour in response to different cytotoxic lesions has been ascribed to the involvement of distinct signalling pathways [38]. However, the ability of drugs that damage microtubules (and therefore cause mitotic arrest) to induce Bcl-2 phosphorylation raises the possibility that the modification reflects a common physiologic process associated with mitotic events [40]. Multiple signal transduction pathways involving specific protein kinases participate in the regulation of cell proliferation, cell cycle progression, mitosis, and apoptosis [31, 41]. There is evidence to suggest that such pathways (in particular, those implicated in the regulation of the cell cycle and apoptosis) may be interconnected [42]. Thus, the complex network among multiple pathways does not allow a univocal interpretation of the cause–effect relationship in the phosphorylation cascade.

However, several lines of evidence show that Bcl-2 phosphorylation results in its inactivation, thus favoring the apoptotic cell death. In some cell systems, the use of phosphatase inhibitors has suggested that Bcl-2 loses its anti-apoptotic function following serine phosphorylation [32, 43]. Bcl-2 phosphorylation by taxol involves multiple sites located in a large unstructured loop domain consisting of about 60 amino acids; serine-70 seems to be a critical site for drug-induced phosphorylation of Bcl-2 [44]. The loop domain of Bcl-2 is required for the modulation of Bcl-2 function [45,46]. Mutant forms of Bcl-2 with an alteration of serine-70 or with deletion of the loop region were unable to be phosphorylated by taxol and were more effective in preventing apoptosis than wild-type Bcl-2 [46]. Multiple kinases have been implicated in the phosphorylation of Bcl-2. They include Raf-1 kinase [47], protein kinase Cα [35], protein kinase A [39], or Jun N-terminal kinase/stress-activated protein kinase [48]. Since Bcl-2 phosphorylation was detected as an early event during drug treatment [40, 49], the question remains whether the event is a determinant of apoptosis. Bcl-2 is normally phosphorylated during mitosis; thus, a plausible interpretation of the role of Bcl-2 in response to antimicrotubule drugs could be that prolonged phosphorylation following drug-induced cell cycle arrest might confer susceptibility to apoptosis to ensure the elimination of damaged cells [49]. Thus, although phosphorylation of Bcl-2 in response to antimitotic agents may be interpreted as a normal process secondary to mitotic arrest, the elevated and persistent mitotic kinase activities could lower the threshold for apoptosis.

Consistent with the interpretation relating Bcl-2 phosphorylation to its inactivation is the recent finding that in a breast carcinoma tumor model, MX-1, DNA-damaging agents (including anthracyclines and platinum compounds) are also able to induce early phosphorylation of Bcl-2 in spite of no evidence of mitotic arrest [21,50]. The tumor model is hypersensitive to cisplatin and to taxanes. Despite the different mechanisms of action, treatment with cisplatin or taxanes achieves complete and persistent tumor regression in all treated animals. A comparative study of agents belonging to the same class but with a variable efficacy against MX-1 tumor indicated an association between drug-induced Bcl-2 phosphorylation and antitumor activity. Among tested DNA-damaging agents, only effective agents (cisplatin and a disaccharide doxorubicin analog) induced a marked Bcl-2 phosphorylation detectable at 24 hr after treatment. The effect was very persistent following treatment with cisplatin, which was the most effective agent against the MX-1 tumor, since a single suboptimal dose was sufficient to achieve complete tumor regression. In contrast, a less effective platinum compound induced a delayed protein phosphorylation. Since a parallelism was observed between Bcl-2 phosphorylation and apoptotic response, we suggested that Bcl-2 phosphorylation is an early signal, likely reflecting Bcl-2 inactivation and favouring the onset of apoptosis [21].

A similar parallelism between apoptosis induction and Bcl-2 phosphorylation by genotoxic agents was found in a small cell lung cancer cell line (unpublished data). In the cell system, Bcl-2 phosphorylation was observed 24 hr after exposure to cytotoxic drug concentrations (Fig. 1) and preceded a marked apoptotic response detectable at 48–72 hr.

However, Bcl-2 phosphorylation is not a general event in response to DNA damage of Bcl-2-expressing tumor cells. As previously reported by others [39], we did not find protein phosphorylation in the MCF-7 breast carcinoma cell

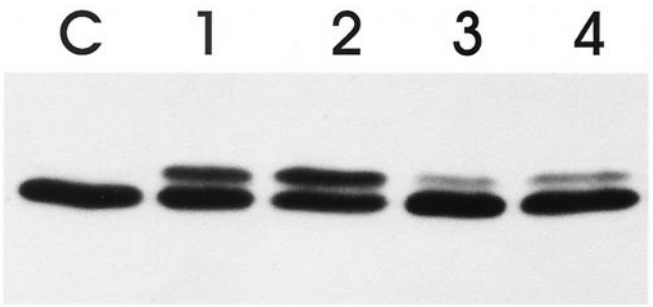

Fig. 1. Bcl-2 phosphorylation in the small cell lung cancer cells, POGB, following treatment with taxol or doxorubicin. Cells were treated for 24 h with cytotoxic concentrations of taxol (1, IC_{50} and 2, $IC_{80,}$ respectively) or with doxorubicin (3, IC_{50} and 4, $IC_{80,}$ respectively).

line after exposure to doxorubicin. Whereas Bcl-2 phosphorylation is a common event in response to all tested antimicrotubule agents probably linked to mitotic arrest [37,39], there is no evidence of a general pathway converging on Bcl-2 in cellular response to genotoxic agents. It is conceivable that the functional significance of Bcl-2 phosphorylation may be somewhat different in response to different cytotoxic stresses. In cells treated with antimicrotubule agents, the convergence of mitotic arrest, microtubule dysfunction, and kinase activation results in Bcl-2 phosphorylation, an event that favours cell death, but it may not be a determinant of chemosensitivity. In contrast, Bcl-2 phosphorylation following DNA damage appears to be a cell-specific event. Presumably, depending on the biological context, different cell death pathways may be activated [2,3,51]. In some cases, Bcl-2-independent pathways may be involved in response to specific DNA lesions [4]. However, when signaling events following drug-induced DNA damage converge on Bcl-2 (e.g. p53-dependent apoptosis as observed in MX-1 breast carcinoma), its phosphorylation is an early signal likely reflecting the onset of drug-induced apoptosis [21].

4. Conclusions

The evidence that survival genes may be deregulated in tumors [3] and the better understanding of their role in the mechanism of apoptosis regulation have provided new insights to develop therapeutic strategies aimed at enhancing cellular susceptibility to drug-induced apoptosis and improving the therapeutic index of cytotoxic therapy. The identification of the biological context in which Bcl-2 may confer a relative resistance to apoptosis induction is expected to better define the therapeutic interest of manipulation of Bcl-2 functions. Post-transcriptional modifications of Bcl-2, related to modulation of its protective function against apoptosis, include phosphorylation and proteolytic cleavage. The latter modification is activated by downstream caspase and contributes to amplification of the caspase cascade [52].

Phosphorylation of Bcl-2, a normal physiologic event during mitosis, is induced by antimitotic agents and likely reflects the onset of apoptosis. If the function of Bcl-2 is critical for the chemosensitivity to agents with different mechanisms of action (e.g. genotoxic agents), a rational combination therapy with antimicrotubule agents may be a promising approach in the treatment of Bcl-2-overexpressing tumors. The evidence that other agents, including drugs targeted to mitochondria [19] and DNA-damaging agents [21], may induce phosphorylation of Bcl-2, presumably through different signalling pathways, suggests the possibility of exploiting novel targets to improve the therapeutic efficacy of conventional agents and circumvent the Bcl-2-mediated resistance to apoptosis. In some tumor cells (e.g. glioma cells), DNA-damaging agents may induce expression of death receptors (e.g. tumor necrosis factor family receptors) or their ligands [53]. Fas-mediated apoptosis may be Bcl-2 independent [3]. However, the evidence that, in such a cellular context, high levels of Bcl-2 may be pro-apoptotic in Fas-mediated cell response [54] suggests a participation of mitochondria in the amplification of the effector step involving caspase activation, since Bcl-2 itself may be converted to a Bax-like death effector [52]. The ability of the mitochondria-targeted drug lonidamine to induce apoptosis and the antagonist effect of Bcl-2 [17,29] indicate that the regulatory step involving Bcl-2 is a promising area in future efforts to rationally manipulate cellular response for therapeutic improvement.

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