

Comparison of the anti-apoptotic effects of Bcr-Abl, Bcl-2 and Bcl-x_L following diverse apoptogenic stimuli

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Received 9 January 2003; revised 28 February 2003; accepted 19 March 2003

First published online 27 March 2003

Edited by Vladimir Skulachev

Abstract Ectopic expression of Bcr-Abl, Bcl-2 or Bcl-x_L in HL-60 cells conferred resistance to apoptosis against a variety of death-inducing agents. Bcr-Abl-mediated interference with mitochondrial events was confirmed by the analysis of the loss of mitochondrial transmembrane potential and cytochrome *c* release. HL-60.Bcr-Abl cells were extremely resistant to all apoptogenic stimuli tested, even in circumstances where HL-60.Bcl-2 or HL-60.Bcl-x_L cells were only partially protected from apoptosis. The levels of Mcl-1, Bax, Bid, Akt, c-IAP-1, c-IAP-2, XIAP and c-FLIP were compared in all HL-60 lines. Our findings show that Bcr-Abl is a more powerful anti-apoptotic molecule than Bcl-2 or Bcl-x_L.

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Key words: Apoptosis; Bcr-Abl; Bcl-2; Bcl-x_L; Mitochondrion

1. Introduction

Apoptosis is a form of cell death triggered during a variety of physiological conditions and is dependent on the activation of particular biochemical pathways inside the dying cells [1]. Once stress signals are initiated, apoptosis can still be halted by the expression of anti-apoptotic molecules of the Bcl-2 family, which play their role at the mitochondrial level by blocking the release of apoptogenic factors such as cytochrome *c*, SMAC/Diablo and AIF [2,3]. Beyond this control point, apoptosis is triggered by the activation of caspase-9 in a multi-molecular complex called apoptosome, which is composed of APAF-1, ATP, cytochrome *c* and pro-caspase-9 molecules. Afterwards, caspase-9 activates the executioner caspases, such as caspase-3, -6 and -7. Although caspases are the molecules responsible for the morphological and biochemical changes associated with apoptosis, the control of the decision between life and death relies on the mitochondria [4,5].

Another checking point where apoptosis can be inhibited is the activation of caspases, which can be blocked by certain

endogenous inhibitors called IAPs (inhibitors of apoptosis). IAPs were first identified in baculoviruses, where they act as a molecular tool for preventing apoptosis in the host insect cells, therefore boosting viral replication. They have multiple biological activities and besides binding and inhibiting caspases they can regulate cell cycle progression and modulate receptor-mediated signal transduction [6,7].

Finally, molecules such as c-FLIP are able to interfere with the apoptotic program initiated by the activation of death receptors, by competing with the initiator caspases (caspase-8 and -10) associated with the Fas receptor complex, shutting off the downstream Fas signaling pathway [8].

Bcr-Abl is a constitutively activated tyrosine kinase responsible for the resistance to apoptosis observed in Philadelphia chromosome-positive leukemia. It has been suggested that Bcr-Abl operates at the mitochondrial level to prevent apoptosis initiated by a variety of chemotherapeutic treatments [9,10]. In fact, we have shown that Bcl-x_L, but not Bcl-2, mediates in part the anti-apoptotic effect of Bcr-Abl [10], although it was also proposed that Bcl-2 may play a role in other experimental systems [11,12]. Recently, it was revealed that Bcr-Abl regulates the transcription of *bcl-x_L* through the activation of STAT-5 [13]. In addition, anti-apoptotic signals initiated by Bcr-Abl may also involve the phosphoinositide 3'-kinase (PI3K)/Akt pathway [14], even though in our experimental system inhibitors such as wortmannin do not interfere with the strong resistance to apoptosis observed in HL-60.Bcr-Abl cells, despite knocking down PI3K activity [15].

The aim of this work was to systematically compare the outcome of ectopic expression of Bcr-Abl, Bcl-2 and Bcl-x_L on the resistance to apoptosis induced by a variety of triggering agents. We therefore used stable lines of transfected HL-60 cells to investigate which step of the apoptotic machinery was most influenced by each of these anti-apoptotic molecules.

2. Materials and methods

2.1. Cells

Human acute myeloid leukemia HL-60 cells ectopically expressing Bcr-Abl, Bcl-2 or Bcl-x_L were previously described [9,10].

2.2. Reagents

The bacterial expression vector pProEX-annexin V was a generous gift from Dr. Seamus J. Martin (Trinity College, Dublin, Ireland). DiOC₆ was purchased from Molecular Probes (Eugene, OR, USA). Actinomycin D (Act-D), cytosine arabinoside (Ara-C), cycloheximide

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Abbreviations: Act-D, actinomycin D; Ara-C, cytosine arabinoside; Cal-C, calphostin C; CHX, cycloheximide; mAb, monoclonal antibody; Noco, nocodazole; pAb, polyclonal antibody; STS, staurosporine; VCS, vincristine sulfate; VP-16, etoposide

(CHX), etoposide (VP-16), nocodazole (Noco), staurosporine (STS) and vincristine sulfate (VCS) were ordered from Sigma Chemicals (St. Louis, MO, USA). Calphostin C (Cal-C) and camptothecin were from Calbiochem (San Diego, CA, USA).

2.3. Antibodies

Antibodies were obtained from different sources. Anti-CD95 IgM monoclonal antibody (mAb) (CH11) was purchased from Medical and Biological Laboratories (Nagoya, Japan). Anti-XIAP mAb, anti-caspase-3, anti-caspase-9, anti-Bax and anti-Bcl-x_L polyclonal antibodies (pAbs) (Pharmingen, San Diego, CA, USA), anti-c-IAP-1 and anti-Mcl-1 pAbs (Santa Cruz, Santa Cruz, CA, USA) anti-phosphotyrosine mAb (Upstate Biotech, Lake Placid, NY, USA), anti-Akt mAb (New England Biolabs, Beverly, MA, USA), anti-c-IAP-2 pAb (R&D Systems, Minneapolis, MN, USA), anti-c-Abl mAb (Calbiochem, San Diego, CA, USA), anti-Bcl-2 mAb (Dako, Carpinteria, CA, USA), anti-actin mAb (ICN, Costa Mesa, CA, USA), anti-mouse IgG-horseradish peroxidase (HRP) and anti-rabbit Ig-HRP (Amersham Pharmacia Biotech, Uppsala, Sweden) were used as suggested by the manufacturers. Anti-caspase-8 mAb and anti-c-FLIP mAbs were generously provided by Dr. Marcus Peter (University of Chicago, Chicago, IL, USA). Anti-Bid mAb was kindly provided by Dr. Stanley Korsmeyer (Washington University, Seattle, WA, USA) and anti-SMAC pAb was a generous gift from Dr. Seamus J. Martin (Trinity College, Dublin, Ireland).

2.4. Production of recombinant annexin V-FITC

Recombinant His-tagged annexin V was produced using the pProEX[®] HT Prokaryotic Expression System (Life Technologies) and purified in a HiTrap[®] Chelating HP column (Amersham Biosciences), according to the instruction of the manufacturer. Purified His-annexin V was labeled with FITC (Sigma-Aldrich), following the protocol provided with the product.

2.5. Determination of apoptosis

Apoptosis was assessed by several criteria. DNA fragmentation was quantified by cell cycle analysis of total DNA content as described by Nicoletti et al. [16]. The collapse of the inner mitochondrial transmembrane potential ($\Delta\psi_m$) was measured using DiOC₆ dye [17,18]. Quantitative determination and differentiation of viable, early, and late apoptotic cells were carried out using annexin V/propidium iodide staining, as previously described [19]. All results represent the average obtained in triplicate samples. The variations among the triplicates were always less than 10%. Every experiment was repeated two to three times.

2.6. SDS-PAGE and Western blot

Protein samples were resolved under reducing conditions as previously described [18,20]. For total cell lysates, cells were harvested, washed once in ice-cold phosphate-buffered saline (PBS), lysed directly in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2.5% 2-mercaptoethanol), and boiled for 5 min. For preparation of cytosolic fractions, cells were washed once with ice-cold PBS and permeabilized for 5 min on ice at a density of 3×10^7 /ml in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.2, 100 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin, containing 250 μ g/ml digitonin). Samples were then centrifuged at $1000 \times g$ for 5 min at 4°C, the supernatants were collected and appropriately diluted with 5 \times SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. A total of 20–30 μ g of protein was loaded per lane and Western blot reactions on polyvi-

nylidene difluoride membranes were detected using enhanced chemiluminescence (ECL, Amersham, Arlington, IL, USA).

3. Results and discussion

3.1. Multi-drug resistance of HL-60 cells expressing anti-apoptotic genes

Although Bcr-Abl has no structural homology with Bcl-2 members, it has been suggested that this oncogenic tyrosine kinase blocks the apoptotic machinery at the mitochondrial level [9,10], resembling therefore the function of anti-apoptotic members of the Bcl-2 family [21]. To gain insight into the molecular basis that controls the resistance to apoptosis induced by Bcr-Abl, we sought to verify whether the ectopic expression of Bcr-Abl, Bcl-2 or Bcl-x_L in HL-60 cells would provide similar anti-apoptotic signals and consequently similar protection to a variety of apoptogenic insults.

Using the DNA fragmentation assay, we confirmed that the stable expression of Bcr-Abl, Bcl-2 or Bcl-x_L in HL-60 cells converted this apoptosis-sensitive line into resistant cells (Table 1). DNA-damaging agents (Ara-C, VP-16, teniposide, camptothecin), protein kinase inhibitors (STS and Cal-C), cytoskeleton-disrupting drugs (VCS and Noco) and protein (CHX) or RNA (Act-D) synthesis inhibitors were used as the apoptogenic stimuli.

Interestingly, albeit resistant to apoptosis, HL-60.Bcr-Abl, HL-60.Bcl-2 and HL-60.Bcl-x_L cells were susceptible to changes in the cell cycle imposed at least by some of the drugs, such that surviving cells appear to be arrested in G2 after treatments with VCS, Noco (Fig. 1) and, to a lesser extent, Cal-C (data not shown).

When we analyzed the results in more detail, we noticed that the HL-60.Bcr-Abl cells were much more resistant than HL-60.Bcl-2 or HL-60.Bcl-x_L cells. In some cases, including STS treatment, the resistance conferred by Bcr-Abl (85% of cells alive) in comparison to Bcl-2 (44%) and Bcl-x_L (46%) was very impressive (Table 1). Such extreme protection could also be seen after the incubation of cells with camptothecin, where 84% of Bcr-Abl cells were protected from apoptosis whereas just 52% of Bcl-2 and 43% of Bcl-x_L cells remained alive after the treatment (Table 1). In both cases, only 2–4% of the HL-60 vector control cells survived the incubation with these drugs.

3.2. Externalization of phosphatidylserine residues

Since we looked at only one of the consequences of apoptosis, and DNA fragmentation is rather a late event in the apoptosis cascade, we decided to investigate whether phosphatidylserine (PS) externalization was also differentially regulated in HL-60 cells overexpressing Bcr-Abl, Bcl-2 and Bcl-x_L. It is well known that PS externalization happens early dur-

Table 1
Differential resistance of HL-60 cells expressing anti-apoptotic genes

	Control	Teniposide	VP16	Camptothecin	Ara-C	STS	Cal-C	CHX	Act-D	VCS	Noco
HL-60.neo	5 \pm 0.5	97 \pm 2.0	91 \pm 0.2	96 \pm 0.2	60 \pm 2.0	98 \pm 0.4	91 \pm 1.0	39 \pm 5.0	90 \pm 3.0	70 \pm 0.3	59 \pm 12
HL-60.Bcr-Abl	7 \pm 1.4	8 \pm 0.1	7 \pm 0.2	16 \pm 0.7	9 \pm 0.1	15 \pm 0.1	8 \pm 1.5	10 \pm 1.0	11 \pm 0.2	10 \pm 0.8	12 \pm 1.5
HL-60.Bcl-2	4 \pm 0.4	19 \pm 0.1	34 \pm 0.1	48 \pm 0.5	20 \pm 2.0	66 \pm 0.9	18 \pm 4.0	21 \pm 1.7	9 \pm 1.4	26 \pm 1.6	20 \pm 2.3
HL-60.BclxL	4 \pm 0.7	30 \pm 1.3	38 \pm 10	57 \pm 6.0	32 \pm 0.1	64 \pm 1.0	8 \pm 0.7	13 \pm 0.4	5 \pm 0.2	28 \pm 2.0	29 \pm 7.0

HL-60, HL-60.Bcr-Abl, HL-60.Bcl-2 and HL-60.Bcl-x_L were treated for 18 h with different death-inducing agents and apoptosis was estimated by cell cycle analysis. Numbers represent the average percentage \pm S.D. of cells with sub-diploid DNA contents. Results show triplicate samples from a representative of three independent experiments.

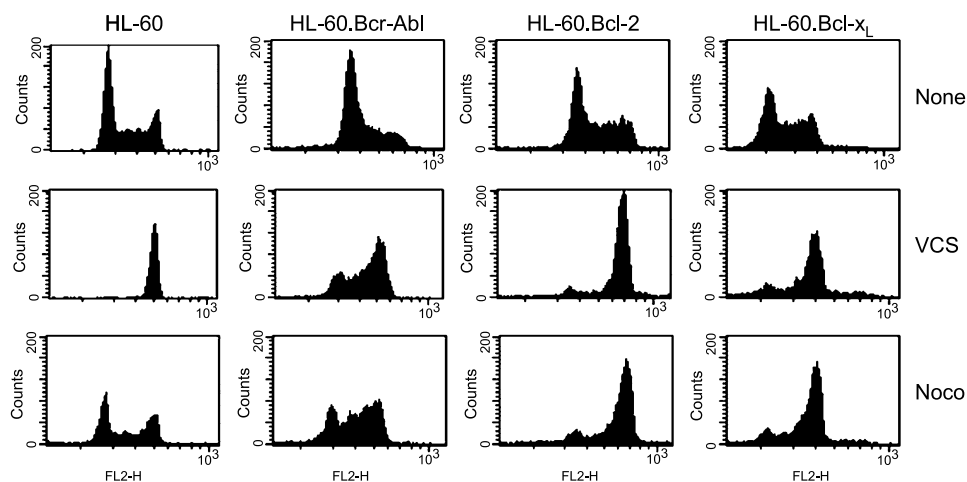


Fig. 1. Changes in cell cycle of HL-60 cells expressing anti-apoptotic genes. HL-60 cell lines were treated with 100 μ M VCS or 10 μ g/ml Noco for 18 h and changes in cell cycle were determined by propidium iodide assay. Figure shows representative data of three independent experiments.

ing apoptosis [19]. Moreover, from a biological point of view this is perhaps the most important modification that occurs in the apoptotic cells, as 'PS flip' is the prominent 'eat-me' signal recognized by macrophages in order to remove the dying cells in the organism without initiating inflammatory reactions [22–24].

PS flip occurred in HL-60 vector control cells after every pro-apoptotic treatment tested, although with different intensities (Fig. 2). HL-60.Bcl-2 and HL-60.Bcl-x_L cells behaved quite similarly regarding to their resistance to PS externalization and HL-60.Bcr-Abl cells displayed the strongest resis-

tance to this apoptotic event, never showing PS flip above control levels (Fig. 2).

3.3. Mitochondrial changes during apoptosis

Most of the apoptogenic signaling pathways are regulated by mitochondrial events such as the release of cytochrome *c* and SMAC/Diablo to the cytosol, which is often accompanied by the loss of mitochondrial transmembrane potential ($\Delta\psi_m$). It is well established in the literature that both Bcl-2 and Bcl-x_L operate in the apoptotic machinery by blocking cell death at the mitochondrial level, therefore preventing the release of

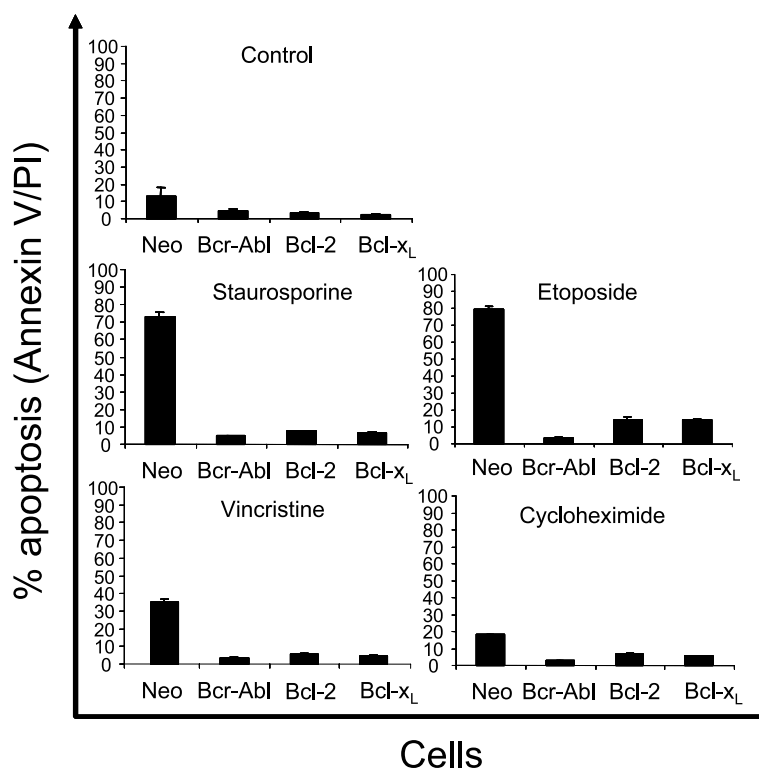


Fig. 2. PS externalization after apoptogenic drugs. HL-60, HL-60.Bcr-Abl, HL-60.Bcl-2 and HL-60.Bcl-x_L were treated for 18 h with different death-inducing agents and apoptosis was estimated by the annexin V binding assay. Numbers represent the average \pm S.D. Figure shows representative data of three independent experiments.

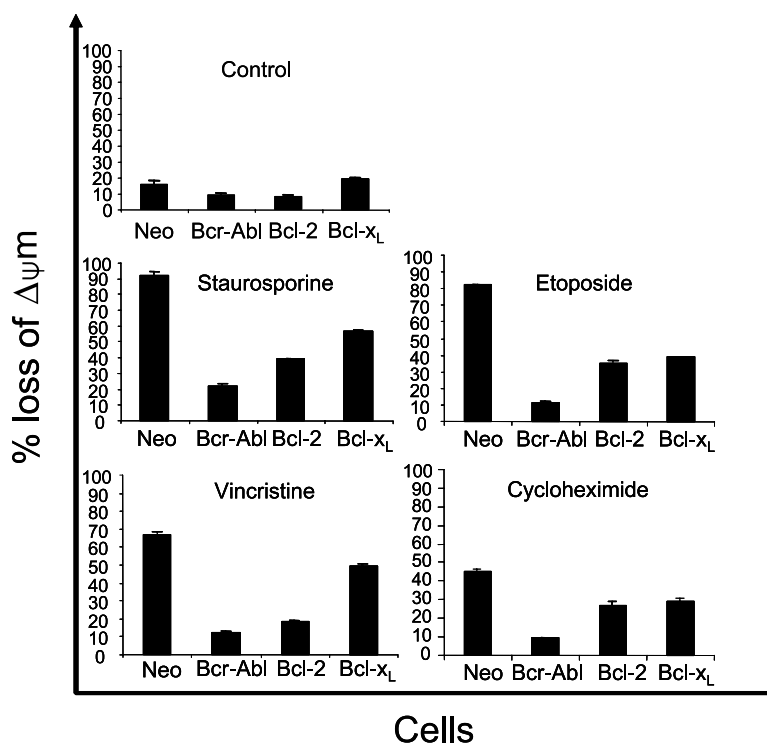


Fig. 3. Loss of $\Delta\psi_m$ during apoptosis. HL-60 clones were treated for 18 h with different death-inducing agents and the collapse of $\Delta\psi_m$ was determined by the DiOC₆ assay. Numbers represent the average \pm S.D. Figure shows representative data of two independent experiments.

cytochrome *c* and the loss of $\Delta\psi_m$ [21]. In extreme situations where the amount of these two molecules is not enough to handle the apoptogenic stimuli, cytochrome *c* is released and death occurs by a caspase-dependent mechanism. Hence, we used four drugs with different modes of action to investigate whether the release of cytochrome *c* and the loss of $\Delta\psi_m$ were differentially affected by the expression of Bcr-Abl, Bcl-2 or Bcl-x_L.

Regarding the changes in $\Delta\psi_m$, we observed that among the lines analyzed, Bcr-Abl-expressing HL-60 cells were again influenced least by pro-apoptotic drugs (Fig. 3). Treatment with STS, VP-16, CHX or VCS induced important losses of $\Delta\psi_m$ in HL-60.neo cells (92%, 82%, 46%, 67%, respectively) and, to a lesser extent, in HL-60.Bcl-2 (39%, 35%, 27%, 19%, respectively) and HL-60.Bcl-x_L (57%, 39%, 29%, 49%, respectively). In contrast, HL-60.Bcr-Abl cells exhibited minimal changes in $\Delta\psi_m$ (22%, 12%, 10%, 13%, respectively), implying that mitochondria from Bcr-Abl-positive cells were more resistant to the deleterious effect of the apoptogenic stimuli. In fact, this presumption was corroborated by the fact that we could not

detect cytochrome *c* translocation from the mitochondria to the cytosol in HL-60.Bcr-Abl cells after the same treatments (Fig. 4 and data not shown). In comparison, only traces of cytochrome *c* were detected in HL-60.Bcl-2 and HL-60.Bcl-x_L cells subjected to similar experimental conditions. As expected, every drug induced cytochrome *c* release in HL-60.neo cells (Fig. 4 and data not shown).

To further confirm that the mitochondrial apoptotic pathway is severely hindered in HL-60.Bcr-Abl cells we examined the activation of caspases-9 and -3 after different apoptogenic stimuli. As we can see, neither caspase-9 nor -3 was activated after VP-16 (Fig. 5) or STS, CHX or VCS (data not shown). We noticed that caspase-8 was activated after caspases-9 and -3 in some circumstances, as a possible positive feedback mechanism. On the other hand, caspase-2 was never activated under our experimental conditions.

Interestingly, Bcr-Abl may also interfere with apoptosis upstream of mitochondria, since the activation of the Fas pathway was blocked in HL-60.Bcr-Abl cells already at the level of caspase-8 activation (Fig. 6A). In accordance with the litera-

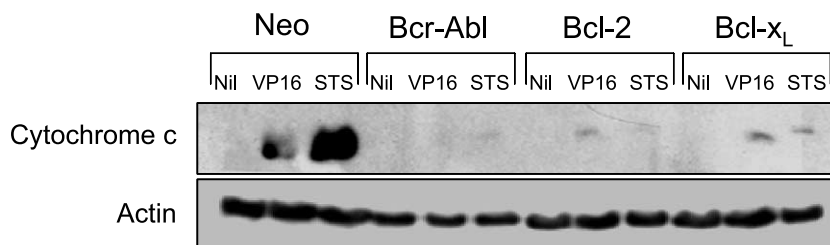


Fig. 4. Cytochrome *c* release in HL-60 cells expressing anti-apoptotic genes. HL-60, HL-60.Bcr-Abl, HL-60.Bcl-2 and HL-60.Bcl-x_L were treated with 1 μ M STS or 10 μ M VP-16 for 4 h and cytosolic extracts were separated by SDS-PAGE and immunoblotted as described in Section 2.

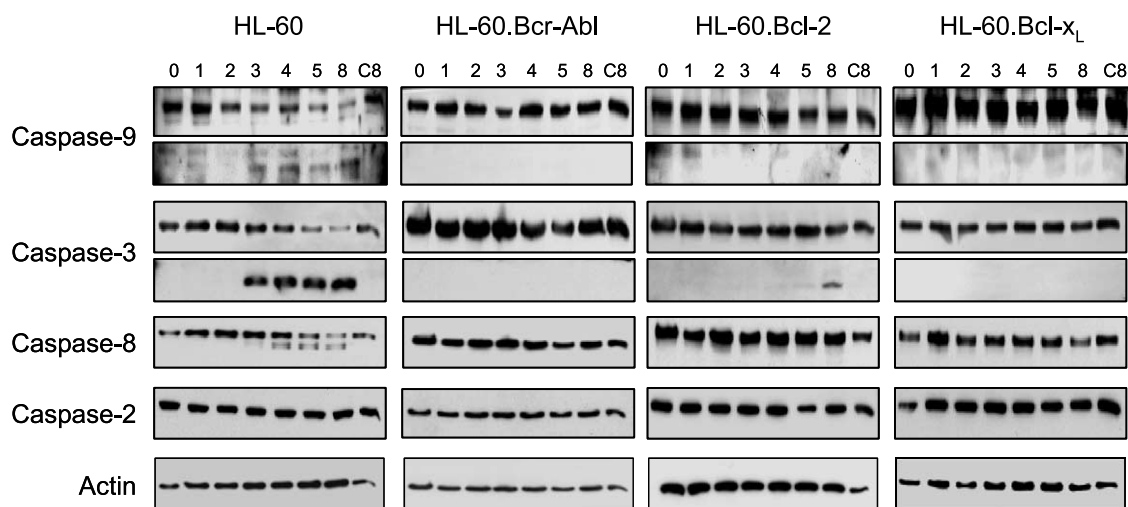


Fig. 5. Caspases are not activated during apoptosis in HL-60 cells expressing anti-apoptotic genes. HL-60, HL-60.Bcr-Abl, HL-60.Bcl-2 and HL-60.Bcl-x_L were treated with 10 μ M VP-16. Total cell lysates were obtained after different periods of time and proteins were analyzed by Western blot using appropriate antibodies.

ture, ectopic expression of Bcr-Abl, Bcl-2 or Bcl-x_L conferred resistance to apoptosis induced by anti-Fas antibodies (Fig. 6B).

3.4. Expression of regulators of apoptosis

We finally compared the expression of some proteins implicated in the regulation of apoptosis in the four cell lines used in this study. Confirming our previous observation [10], while HL-60 cells express Bcl-2 but not Bcl-x_L, HL-60.Bcr-Abl cells express Bcl-x_L but not Bcl-2 (Fig. 7). As expected, the level of Bcl-2 was higher in HL-60.Bcl-2 cells whereas the level of Bcl-x_L was greater in HL-60.Bcl-x_L. The levels of the anti-apoptotic molecules Mcl-1 and the pro-apoptotic protein Bax were similar in all four cell lines. In contrast, the expression of Bid was considerably reduced in HL-60.Bcr-Abl cells, which may therefore contribute to the resistance to apoptosis observed in

these cells. However, it is important to remark that Bid does not play a role in every form of apoptosis. Thus, the reduced level of this pro-apoptotic molecule in HL-60.Bcr-Abl cells cannot entirely explain the outstanding resistance to apoptosis conferred by the ectopic expression of Bcr-Abl in these cells. We were not able to detect the expression of Bad in any of the HL-60 lines. Due to the fact that HL-60 cells are feeble in Src family kinases, the level of phosphotyrosine-containing proteins in these cells is below the sensitivity of our Western blot assay. Therefore, as predicted, phosphotyrosine-containing proteins were found only in HL-60.Bcr-Abl cells. Actin was used as an internal control for the immunoblots in order to certify that the same amount of protein was loaded to the gels in each sample.

The IAP family of proteins comprises caspase inhibitors that were shown to have an evolutionarily conserved role in

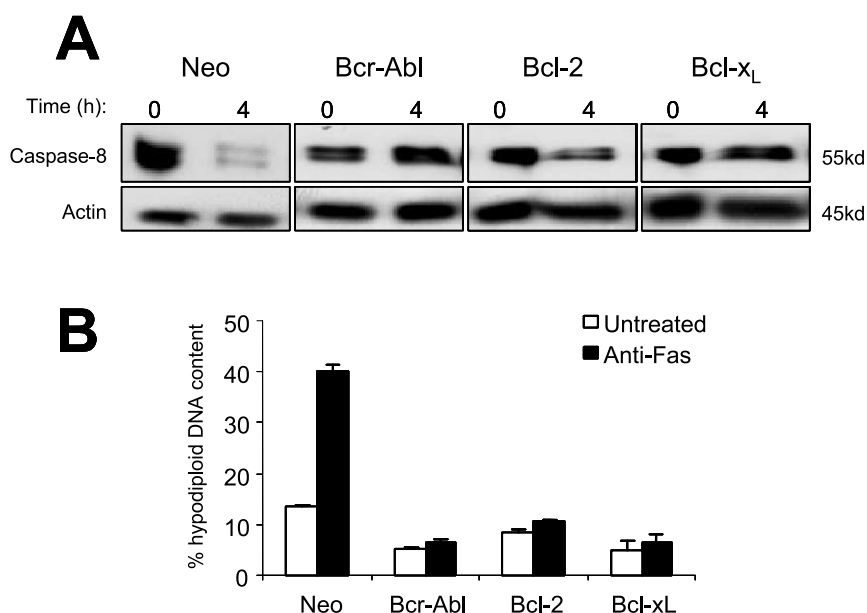


Fig. 6. Impaired caspase-8 activation and apoptosis in HL-60.Bcr-Abl cells treated with anti-Fas antibodies. HL-60 cell lines were incubated for 4 h (A) or overnight (B) in the presence or absence of 1 μ g/ml of anti-Fas antibody (CH11). Western blot analysis of caspase-8 activation (A) and cell cycle analysis of genomic DNA content (B) were performed as described in Section 2.

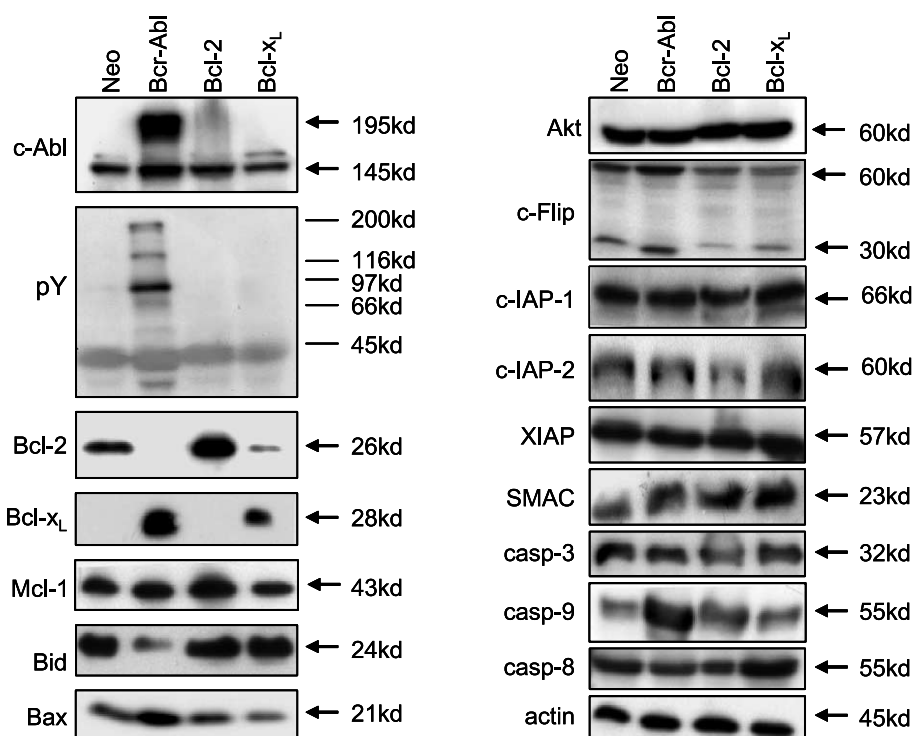


Fig. 7. Expression of proteins involved in apoptosis by HL-60 cells expressing anti-apoptotic genes. Total cell lysates of untreated HL-60, HL-60.Bcr-Abl, HL-60.Bcl-2 and HL-60.Bcl-x_L cells were analyzed by Western blot as described in Section 2.

regulating apoptosis [6,7]. Ectopic expression of human IAP proteins can suppress cell death induced by a variety of stimuli. We therefore investigated the levels of three members of the IAP family, namely c-IAP-1, c-IAP-2 and XIAP, and found that they are similar in all four lines investigated (Fig. 7).

It was recently shown that the anti-apoptotic activity of the IAPs can be obstructed by a mitochondrial molecule named SMAC/Diablo, which is translocated to the cytosol during apoptogenic stimulation [25,26]. Although we still do not know whether Bcr-Abl controls the release of SMAC from the mitochondria to the cytosol in a particular manner, we detected similar amounts of this protein in all cell lines (Fig. 7).

The serine-threonine kinase Akt/PKB was shown to mediate some of the anti-apoptotic activities of survival factors such as platelet-derived and nerve growth factors and insulin-like growth factor-1 [27]. This pathway is initiated by the activation of PI3K after the engagement of survival factor receptors and leads, among other biochemical modifications, to the phosphorylation of Bad, one of the pro-apoptotic members of the Bcl-2 family [28]. Phosphorylated Bad binds to the adapter protein 14-3-3 and remains sequestered in the cytosol. When the survival signal ceases, Bad is dephosphorylated, detaches from the 14-3-3 complex and migrates to the mitochondria where it will cause apoptosis. It has been suggested that Bcr-Abl can activate the PI3K/Akt survival factor pathway, which would consequently sustain the resistance to apoptosis in Bcr-Abl-positive cells [14]. Therefore, we looked at the levels of Akt in the four HL-60 lines and found that they are quite similar (Fig. 7). Although it is obvious that the expression level of this protein does not necessarily correlate with its activity, which may still be different in each of the HL-60 lines, our previous data suggest that the PI3K/Akt

pathway is not activated by Bcr-Abl in HL-60.Bcr-Abl cells and inhibitors of PI3K do not interfere with the resistance to apoptosis found in these cells [15]. To finally determine the contribution of Akt to the resistance of HL-60.Bcr-Abl cells we are at the moment generating an HL-60 cell line overexpressing an active form of Akt as well as an HL-60.Bcr-Abl line that expresses a dominant negative form of Akt.

Another molecule with anti-apoptotic features is c-FLIP, a protein homologous to the caspases but without their catalytic activity [8]. c-FLIP seems to act by competing with caspase-8 to the Fas or other death receptor complexes. Interestingly, the expression of c-FLIP long and short was slightly elevated in HL-60.Bcr-Abl cells compared to the other cell lines (Fig. 7). Although we have not approached this question at the moment, this result may be related to the observation that caspase-8 was not activated in HL-60.Bcr-Abl cells after 4 h incubation with anti-Fas antibodies (Fig. 6).

Knowing that apoptotic cell death is coordinated by certain members of the caspases [1], we looked into the expression of three different caspases. Our findings revealed that the levels of caspases-8 and -3 were similar in all four cell lines (Fig. 7). Surprisingly, HL-60.Bcr-Abl cells seem to express higher levels of caspase-9.

To sum up, we found that the expression of Bcr-Abl in HL-60 cells confers a condition of extreme resistance to apoptosis regardless of the apoptogenic stimuli. Although the mitochondrial pathway is clearly involved in the forms of apoptosis investigated in this study, the resistance of Bcr-Abl-positive cells was stronger than the resistance observed after overexpression of Bcl-2 or Bcl-x_L. In this regard, we found that mitochondria from HL-60.Bcr-Abl cells were remarkably immune to the deleterious effect of the apoptogenic stimuli. In addition, Bcr-Abl was capable of protecting HL-60 cells in situations where Bcl-2 or Bcl-x_L has no or very little effect.

Finally, the expression of Mcl-1, Bad, Bax, c-IAP-1, c-IAP-2, XIAP and Akt was similar in all HL-60 cell lines and, therefore, none of these molecules could be responsible for the anti-apoptotic effect of Bcr-Abl.

Acknowledgements: We wish to thank Drs. Marcus Peter, Stanley Korsmeyer and Seamus Martin for the generous gifts of antibodies. We appreciate the technical assistance of Ulisses R. da Silva and Paula C.O. Gorgueira in the purification of recombinant annexin V and preparation of annexin V-FITC. G.B., R.W. and C.F.C. were recipients of M.Sc. fellowships from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). M.Y. was the recipient of a M.Sc. fellowship from the Brazilian Science National Council (CNPq). This work was supported by CNPq and FAPESP.

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