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Differential regulation of apoptosis in AK-5 tumor cells by the proto-oncogene Bcl-2: presence of Bcl-2 dependent and independent pathways

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Abstract The anti-apoptotic protein Bcl-2 functions as a crucial negative regulator of apoptosis. Bcl-2 has been shown to prevent the efflux of apoptogenic factors from mitochondria to cytosol, thus inhibiting cell death. Here, we show the susceptibility of a spontaneously regressing, rat histiocytic tumor cell line, AK-5, to the apoptotic effects of diverse stimuli and the ability of Bcl-2 overexpression to block cell death. Bcl-2 overexpression selectively inhibits apoptosis induced by ceramide and serum factor from AK-5 tumor regressing animals but not actinomycin D and curcumin, whereas the pancaspase inhibitor z-Val-Ala-Asp fluoromethylketone completely blocks apoptosis, irrespective of the inducer used. The ability of Bcl-2 overexpression to block cell death does not depend on its ability to prevent cytochrome *c* release but correlates with its ability to prevent the dissipation of mitochondrial transmembrane potential. The results demonstrate that there are inducer dependent redundant activation pathways in a single cell, which may either be Bcl-2 dependent or independent.

Key words: AK-5 cell; Apoptosis; Bcl-2; Cytochrome *c* release

1. Introduction

The Bcl-2 family of proteins constitutes one of the most crucial classes of apoptosis-regulatory proteins. These include both the pro-apoptotic (Bax, Bad, Bak, Bid, Bcl-Xs, Bim, Bik) and anti-apoptotic (Bcl-2, Bcl-X_L, Bcl_w, Mcl_1) proteins [1,2]. These proteins are mostly localized to the outer membranes of mitochondria, endoplasmic reticulum and nucleus, as a result of a carboxy-terminal membrane anchor [3]. Members of this family are capable of dimerization and the ratio of pro- to anti-apoptotic molecules determines, in part, the susceptibility of cells to undergo programmed cell death [4]. The proto-oncogene Bcl-2 was originally isolated from human follicular lymphomas harboring a t(14;18) chromosomal translocation [5]. It is found primarily at the outer–inner membrane contact sites of mitochondria, where permeability transition (PT) pores are expected to form [6]. Bcl-2 inhibits cell death in response to diverse stimuli [2] and inhibits both the mitochondrial and nuclear manifestations of apoptosis. The precise mechanism by which Bcl-2 modulates cell death is not clearly understood but its anti-apoptotic functions are attributed to the reduction of reactive oxygen species levels [7], effect on the mitochondrial proton flux and modulation of mitochondrial calcium homeostasis [8,9]. Bcl-2 also inhibited the release of cytochrome *c* and an apoptogenic protease from the inner membrane space of mitochondria into the cytosol, thus preventing caspase activation and cell death [10–12]. However both the mechanisms of cytochrome *c* redistribution and its regulation by Bcl-2 remain controversial.

Work in our lab is focussed on deciphering the molecular mechanisms responsible for the spontaneous regression of AK-5 tumor, a rat histiocytoma [13]. We have earlier shown the ability of the serum factor from tumor regressing animals to induce apoptosis in AK-5 cells in vitro. In the present study, we show the susceptibility of AK-5 tumor cells to the apoptotic effects of serum factor from tumor regressing animals, curcumin (a pigment derived from the rhizome of *Curcuma longa*), actinomycin D (transcriptional inhibitor) and ceramide (the pleiotropic lipid second messenger) [14–16] and the regulation of cell death in these cells by Bcl-2.

2. Materials and methods

2.1. Animals, tumor and cell culture

AK-5 tumor was maintained as ascites in an inbred colony of Wistar rats by injecting 5×10^6 tumor cells intraperitoneally (i.p.) [13]. Animals which were injected with tumor cells (5×10^6) subcutaneously (s.c.) and had rejected the tumor were used as the source for anti-AK-5 antiserum (serum factor). We have used a single cell clone of AK-5 adapted to grow in vitro, called BC-8 in all the studies, to avoid ambiguity in results due to tumor heterogeneity [17]. BC-8 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum in an atmosphere of 95% air and 5% CO_2. Cell viability was assessed by trypan blue dye exclusion assay.

2.2. Induction of apoptosis and fluorescence microscopy

After treatment with the serum factor for appropriate time, cells were washed with phosphate-buffered saline (PBS), and fixed in 80% methanol. Cells were stained with 1 μg/ml propidium iodide (PI) reagent (Calbiochem), and observed under a fluorescence microscope (Nikon Optiphot). Apoptotic cells were identified based on the fragmented nuclear morphology.

2.3. Subcellular fractionation and Western blotting

Extraction of mitochondria and cytosol was done according to the previously published procedure [18]. Briefly, BC-8 cells (5×10^6), at

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Abbreviations: zVAD-fmk, z-Val-Ala-Asp fluoromethylketone; PT, permeability transition; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; $\Delta\psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3′,3′-dihexyloxacarbocyanine

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the end of the treatment, were washed with ice-cold PBS, and resuspended in 50 μl of buffer A (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol) containing 250 mM sucrose and protease inhibitors (1 mM PMSF, 1 μg/ml each of leupeptin, aprotinin, pepstatin and chymostatin). The cells were homogenized by giving 40 strokes in a glass homogenizer using a B type pestle. Unlysed cells and nuclei were removed by centrifuging the supernatants at $1000\times g$ at 4°C for 10 min. The resulting supernatant was subjected to $10\,000\times g$ centrifugation at 4°C for 20 min. The pellet fraction (mitochondria) was washed with buffer A, and solubilized in TNC buffer (10 mM Tris–acetate, pH 8.0, 0.5% NP-40, 5 mM $CaCl_2$). The supernatant was recentrifuged at $100\,000\times g$ (4°C, 1 h) to generate cytosol. Protein concentration was determined by Lowry's method. 25 μg of pellet fractions and 50 μg of cytosolic fractions were subjected to 12.5% SDS–PAGE. Separated proteins were transferred to a nitrocellulose membrane and probed with 1:1000 dilution of a monoclonal antibody to cytochrome *c* (Pharmingen, San Diego, CA, USA). Mitochondrial contamination of cytosolic extracts was determined by probing the blot with mouse anti-cytochrome *c* oxidase subunit IV (Molecular Probes Inc., Eugene, OR, USA). The blots were treated with an appropriate secondary antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and developed using NBT and BCIP.

2.4. Transfection of BC-8 cells with Bcl-2 gene

BC-8 cells (2×10^6) were transfected with the linearized pMEP4 vector, with and without full length murine Bcl-2 gene by the electroporation method described earlier [19]. The transfected clones were selected with hygromycin B (400 μg/ml) for 14 days. Single cell clones were obtained by limiting dilution procedure. Clones were screened by Northern hybridizations and immunofluorescence assay (data not shown) and the positive clones were expanded and used in these studies.

2.5. Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$)

Mitochondrial energization was determined as the retention of the dye 3′,3′-dihexyloxacarbocyanine, $DiOC_6(3)$ (Molecular Probes Inc., Eugene, OR, USA). BC-8 cells (1×10^6) at different time points of apoptotic induction were washed with PBS, and treated with 50 nM $DiOC_6(3)$ at 37°C for 30 min. The cells were washed and the quantitation of cells retaining $DiOC_6(3)$ was done in a FACStar PLUS Cytofluorometer (Becton-Dickinson, San Jose, CA, USA), using the Cell-Quest software. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP; 50 μM) was used as a positive control for maximum $\Delta\psi_m$ disruption.

Mitochondrial membrane potential was also analyzed by confocal laser scanning microscopy on a Meridian Ultima Scan head attached to an Olympus IMT-2 inverted microscope after staining cells with $DiOC_6(3)$ and Hoechst (1 μg/ml).

3. Results

3.1. Induction of apoptosis in AK-5 cells by different stimuli

AK-5 tumor, a rat histiocytoma, is being maintained as ascites in an inbred colony of Wistar rats. When injected i.p., AK-5 tumor is highly malignant and kills all the animals, but s.c. transplantation results in solid tumors which regress spontaneously in 80% of the animals. The animals which had rejected the tumor were immune to further challenges of the tumor by either route, and the serum from these animals had a potent apoptosis inducing ability on the AK-5/BC-8 cells in vitro.

Table 1
Quantitative determination of apoptosis in BC-8 cells[a]

Group	Apoptotic cells (%)
1. Control BC-8 cells	5.3
2. BC-8 cells+serum factor	69.0
3. BC-8 cells+curcumin (25 μM)	80.3
4. BC-8 cells+actinomycin D (1 μg/ml)	75.9
5. BC-8 cells+ceramide (50 μM)	65.5

[a]BC-8 cells were treated with various inducers for 12 h, fixed and stained with PI. The values are representative of three independent experiments.

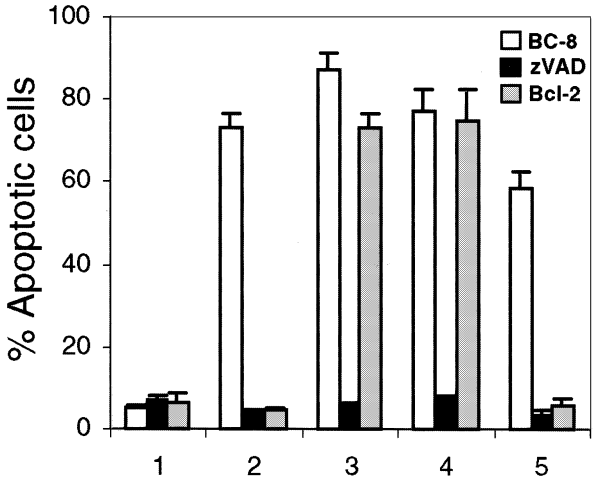

Fig. 1. Differential pattern of inhibition of apoptosis in BC-8 cells overexpressing Bcl-2 or pretreated with zVAD-fmk. Bcl-2 overexpressing cells and BC-8 cells pretreated with zVAD-fmk (100 μM) for 1 h were induced for 12 h with serum factor (2), 25 μM curcumin (3), 1 μg/ml actinomycin D (4), 50 μM C8-ceramide (5). (1) represents untreated cells. Cells were fixed and stained with PI. Data represent mean ± S.E.M. of three independent experiments.

We have seen the susceptibility of BC-8 cells to the apoptotic effects of the serum factor (10% v/v) from tumor regressing animals as well as curcumin (25 μM), the synthetic C8-ceramide (50 μm) and actinomycin D (1 μg/ml). BC-8 cells upon induction of apoptosis with these agents underwent all the classical features of apoptosis with exquisite synchrony like chromatin and cytoplasmic condensation, membrane blebbing and DNA fragmentation. Most of the cells were rendered apoptotic by 12 h of treatment as seen by PI staining (Table 1).

3.2. Inhibition of apoptosis by Bcl-2 overexpression is inducer dependent

Apoptosis induced in BC-8 cells by different apoptotic stimuli proceeds with the activation of caspase-3. In order to ascertain the involvement of caspases during apoptosis, BC-8 cells were pretreated with a broad spectrum, cell permeable inhibitor of caspases, z-Val-Ala-Asp fluoromethylketone (zVAD-fmk) (100 μm), for 30 min, followed by induction with serum factor, curcumin, actinomycin D and C8-ceramide. zVAD treatment completely inhibited the appearance of apoptotic morphology and DNA fragmentation as assessed by PI staining (Fig. 1). Surprisingly Bcl-2 overexpression inhibited apoptotic cell death induced by serum factor and ceramide and the cells proliferated normally whereas Bcl-2 overexpression had no effect in cells treated with actinomycin D and curcumin.

3.3. The ability of Bcl-2 overexpression to block cell death does not correlate with its ability to prevent cytochrome c release

Apoptosis in BC-8 cells induced with all the four apoptotic agents proceeds with the translocation of cytochrome *c* from mitochondria to cytosol. Western analysis was done to detect

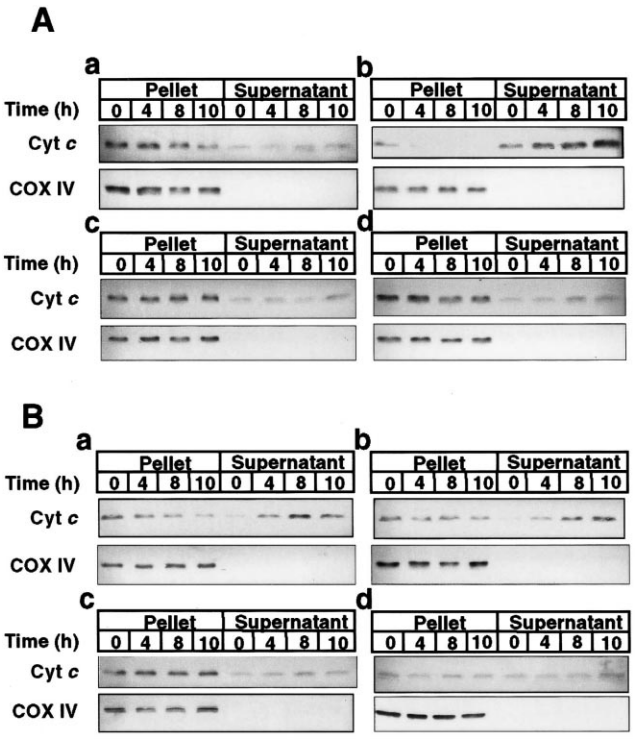

Fig. 2. Bcl-2 overexpression does not prevent cytochrome *c* efflux in BC-8 cells. (A) Western blot analysis showing the time bound release of cytochrome *c* into the cytosol. Apoptosis was induced in BC-8 cells by the serum factor (a), 25 μM curcumin (b), 1 μg/ml actinomycin D (c) and 50 μM C8-ceramide (d). (B) Cytochrome *c* translocation is also shown by Western analysis of mitochondrial and cytosolic fractions of Bcl-2 overexpressing cells induced with the serum factor (a), 25 μM curcumin (b), 1 μg/ml actinomycin D (c) and 50 μM C8-ceramide (d). COXIV staining in (A) and (B) serves as loading control and also rules out the possibility of mitochondrial contamination in the cytosol.

the levels of cytochrome *c* in both soluble cytosolic and the mitochondria-enriched, heavy membrane fractions (the pellet), obtained after treatment of cells with different inducers of apoptosis. The results showed a significant efflux of this protein from mitochondria to cytosol, very early during apoptosis, which increased with time, reaching a peak at 10 h with a concomitant decrease in the immunoreactivity of the pellet fraction (Fig. 2A). The absence of cytochrome *c* oxidase (subunit IV) in the supernatant fraction rules out the possibility of mitochondrial contamination in cytosolic extract preparations (Fig. 2A).

With a view to investigate whether the inhibitory potential of Bcl-2 overexpression reflects its ability to prevent cytochrome *c* release, Western analysis of both the cytosolic and the pellet fraction of Bcl-2 overexpressing cells induced with all the four apoptotic agents was done. As shown in Fig. 2B, there was no correlation between the inhibition of cell death by Bcl-2 and cytochrome *c* release. Cytochrome *c* efflux was seen irrespective of the ability or inability of Bcl-2 to block cell death.

3.4. *The inducer dependent ability of Bcl-2 overexpression to inhibit apoptosis correlates with its ability to prevent $\Delta\psi_m$*

Loss of $\Delta\psi_m$ is a late event during apoptosis in BC-8 cells, which paralleled DNA fragmentation. We have monitored the mitochondrial membrane potential of BC-8 cells by flow cytometry, using the mitochondrial potential-sensitive dye DiO-$C_6(3)$. Treatment of cells with all the four apoptotic inducers led to the dissipation of mitochondrial membrane potential by 8 h of treatment (Fig. 3A). Cells treated with 50 μM CCCP, a protonophore, resulted in complete collapse of $\Delta\psi_m$, suggesting that the uptake of $DiOC_6(3)$ depends on the H^+ gradient in mitochondria.

In an effort to investigate whether inhibition of cell death by Bcl-2 correlates with its ability to prevent the dissipation of $\Delta\psi_m$, Bcl-2 overexpressing clone was treated with different apoptotic inducers for 8 h, stained with $DiOC_6(3)$ and the $\Delta\psi_m$ was quantitated by flow cytometry. Fig. 3B shows that overexpression of Bcl-2 prevented the loss in $\Delta\psi_m$ in cells treated with the serum factor and ceramide (panels b, e), whereas in Bcl-2 cells treated with curcumin and actinomycin D, there is dissipation of $\Delta\psi_m$, resulting in cell death (panels c, d). The uptake of the potential-sensitive dye was also examined by dual staining of cells with $DiOC_6(3)$ and Hoechst followed by confocal analysis, which showed a correlation between perturbation in $\Delta\psi_m$ and cell death (Fig. 4A). Fig.

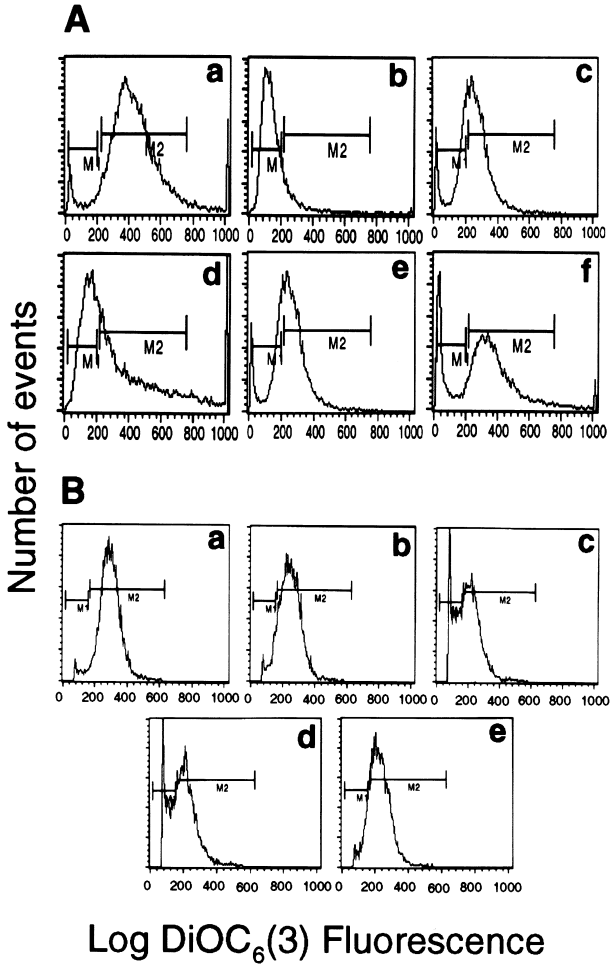

Fig. 3. Quantitation of $\Delta\psi_m$ by $DiOC_6(3)$ staining by flow cytometry. A: (a) represents untreated BC-8 cells; (c), (d), (e) and (f) represent BC-8 cells treated with the serum factor, 25 μM curcumin, 1 μg/ml actinomycin D and 50 μM ceramide respectively. As a positive control for the reduction in $\Delta\psi_m$, cells were treated with CCCP (50 μM), a mitochondrial uncoupling agent (b). B: (a) represents untreated Bcl-2-transfected cells, (b), (c), (d) and (e) represent Bcl-2 cells treated with the serum factor, 25 μM curcumin, 1 μg/ml actinomycin D and 50 μM ceramide respectively.

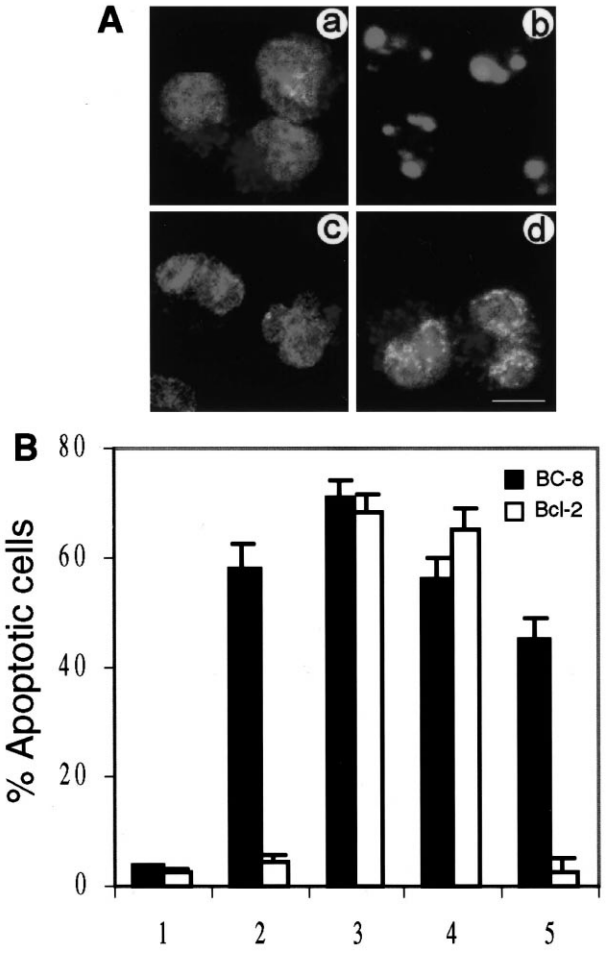

Fig. 4. Determination of $\Delta\psi_m$ disruption by confocal microscopy. A: BC-8 cells upon induction of apoptosis for 8 h by serum factor (b) (shown as a representative) were stained with 50 nM $DiOC_6$ and counter-stained with Hoechst (1 μg/ml), followed by confocal microscopy. (d) represent Bcl-2 overexpressing cells induced with serum factor. (a) and (c) represent untreated BC-8 cells and BC-8 cells treated with 50 μm CCCP. Images were obtained by confocal analysis of a 0.2 μm optical section. Scale bar represents 10 μm. B: Quantitation of apoptosis in BC-8 and Bcl-2-transfected cells by PI staining and fluorescence microscopy after cells were induced for 8 h by serum factor (2), 25 μM curcumin (3), 1 μg/ml actinomycin D (4) and 50 μM C8-ceramide (5). (1) represents untreated cells. Data represent mean ± S.E.M. of three independent experiments.

4B shows the extent of cell death in both untransfected and Bcl-2-transfected cells at 8 h post-induction, the time at which the state of mitochondrial integrity was determined.

4. Discussion

Mitochondria have been shown to be the control point in the mainstream of apoptotic cascade in many cell types [20] as well as in cell-free systems [21]. Apoptosis in the majority of cells studied proceeds with the translocation of apoptogenic factors such as cytochrome *c* [10,11,22] and apoptosis inducing factor [12] from mitochondria to the cytosol. In the cytosol, cytochrome *c* forms a complex with Apaf-1 and caspase-9, leading to the activation of caspase-3 [23]. The mechanism by which cytochrome *c* is released into the cytosol is controversial but seems to be regulated in part by the Bcl-2 family of proteins [10,11]. The relative expression levels of the Bcl-2 proteins regulate cell death by controlling mitochondrial homeostasis. The anti-apoptotic protein Bcl-2 prevents programmed cell death in response to a wide variety of stimuli, but the biochemical mechanism governing its cytoprotective effect is not clearly established. The ectopic expression of the pro-apoptotic protein Bax was shown to trigger cytochrome *c* release from mitochondria, in the absence of any death stimulus [24]. The death protective effect of Bcl-2 was linked to its ability to prevent the efflux of cytochrome *c* and caspase activation [10,11]. Bcl-2 has also been shown to inhibit the ion-conducting channel forming activity of Bax in vitro [25].

To investigate the role of Bcl-2 in regulating mitochondrial function, we explored the alterations in mitochondrial physiology that follow diverse apoptotic stimuli and the ability of Bcl-2 to counteract these changes in a single cell type.

Curcumin is the active component of turmeric used commonly as a spice and has been shown to exhibit anti-mutagenic and anti-carcinogenic activities in addition to anti-inflammatory activities [26,27]. Curcumin was highly potent in inducing all the classical manifestations of apoptotic cell death in AK-5 cells.

Ceramide, a product of sphingomyelin hydrolysis, is considered as a lipid second messenger with pleiotropic effects [16]. Increased levels of ceramide have been correlated with terminal differentiation and apoptotic processes [28]. Actinomycin D, an inhibitor of macromolecular synthesis, induces apoptosis in AK-5 cells at a concentration which inhibits RNA synthesis, suggesting that cell death may result due to the inhibition of synthesis of some regulatory proteins which are required for maintaining cell survival and which prevent triggering of cell death in AK-5 cells.

Apoptosis induced in AK-5 cells by all the four stimuli involved the activation of caspases and the redistribution of cytochrome *c* to the cytosol, very early during apoptosis. As opposed to the earlier studies, which demonstrated a disruption in $\Delta\psi_m$ to be an early event, resulting in apoptosis [29], our studies indicate that the disruption of $\Delta\psi_m$ was a late event which paralleled DNA fragmentation representing the point of no return in apoptosis, and the efflux of cytochrome *c* is not a consequence of the disruption in $\Delta\psi_m$.

Bcl-2 had a differential effect in the regulation of apoptosis in AK-5 cells induced with diverse stimuli. Overexpression of Bcl-2 completely blocked apoptosis induced by the serum factor and ceramide, and the cells proliferated normally, whereas it had no effect on apoptosis induced by actinomycin D and curcumin. The cytoprotective ability of Bcl-2 was not a consequence of the inhibition of the cytochrome *c* release as reported earlier [10,11], as cells induced by all the inducers underwent cytochrome *c* redistribution to the cytosol. The depletion of cytochrome *c*, a protein obligatory for cell survival, had no effect on the viability of cells, treated with serum factor and ceramide. Recent studies demonstrated that the translocation of cytochrome *c* is a reversible event with the depleted cells acquiring cytochrome *c* by de novo protein synthesis [30]. Consistent with our findings, it was shown that microinjection of cytochrome *c* in cells resulted in apoptosis that could not be inhibited by Bcl-X_L expression [31].

Mitochondrial transmembrane depolarization, presumed to be mediated by PT, was shown to irreversibly commit cells to undergo apoptosis, even when the apoptosis inducing stimulus is removed [29]. With the evidence that Bcl-2 can block mi-

tochondrial PT induced by treatment of isolated mitochondria with a variety of agents [32], we investigated whether the differential inhibitory potential of Bcl-2 correlates with its ability to regulate $\Delta\psi_m$ disruption.

Bcl-2 overexpression prevented $\Delta\psi_m$ disruption in cells treated with the serum factor and ceramide, but could not rescue $\Delta\psi_m$ in cells induced by curcumin and actinomycin D. This may reflect different systemic levels of damage of mitochondrial membrane integrity and subsequent disruption of mitochondrial function, which may or may not be overcome by Bcl-2 overexpression.

It is also demonstrated earlier that apoptosis in some types of cells triggered by Fas (CD 95) and certain members of the tumor necrosis factor family of death receptors runs via a Bcl-2 independent pathway, circumventing the participation of mitochondria [33,34]. The ability of Bcl-2 to differentially protect AK-5 cells against apoptosis can be explained by postulating that there are additional parallel pathways that are related, but independent of Bcl-2, and Bcl-2 prevents apoptosis by virtue of its PT-inhibitory potential.

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