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Inhibitory Effects of Bcl-2 on Mitochondrial Respiration

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Summary

In contrast to the well-established anti-apoptotic effect of Bcl-2 protein, we have recently demonstrated that Bcl-2 overexpression by vaccinia virus causes apoptosis in BSC-40 cells, while it prevents apoptosis in HeLa G cells. Given the key role of mitochondria in the process of apoptosis, we focused on effects of Bcl-2 expression on mitochondrial energetics of these two cell lines. In this study we present data indicating that BSC-40 cells derive their ATP mainly from oxidative phosphorylation whereas HeLa G cells from glycolysis. More importantly, we show that in both cell lines, Bcl-2 inhibits mitochondrial respiration and causes a decrease of the ATP/ADP ratio. However, it appears that BSC-40 cells cannot sustain this decrease and die, while HeLa G cells survive, being adapted to the low ratio of ATP/ADP maintained by glycolysis. Based on this observation, we propose that the outcome of Bcl-2 expression is determined by the type of cellular ATP synthesis, namely that Bcl-2 causes apoptosis in cells relying on oxidative phosphorylation.

Key words
Bcl-2 • Apoptosis • ATP • Mitochondrial respiration

Introduction

A variety of physiological death signals as well as pathological cellular insults trigger the genetically programmed pathway of apoptosis (Kerr *et al.* 1972). Bcl-2 is the founding member of a family of proteins that regulate this process of cell death. Originally isolated from the t(14;18) chromosomal breakpoint in human B cell lymphomas, Bcl-2 has since been established to be a proto-oncogene that prolongs cell survival by inhibiting apoptosis (Bakhshi *et al.* 1985). Many members of the Bcl-2 family, such as Bcl-2, Bcl-xL, Bcl-w are inhibitors of cell death, while others, such as Bax, Bad, Bak, Bid or Bim, promote cell death. Homeostasis is maintained in normal tissues by the antagonistic interaction of the anti- and pro-apoptotic Bcl-2 family proteins (Oltvai *et al.* 1993) mainly through the interaction of their so-called BH domains (Bcl-2 homology domains). All members of the Bcl-2 family possess at least one of four of these conserved motifs (BH1 to BH4) (Adams and Cory 1998). In addition to the regulation of apoptosis by heterodimerization of anti-apoptotic and pro-apoptotic members of the Bcl-2 family, some protein members have been suggested to regulate apoptosis independently of each other (Adams and Cory 2001). Bcl-2 is a membrane protein that has been shown to be localized in outer mitochondrial, endoplasmic reticulum and perinuclear membranes (Hockenbery *et al.* 1990).

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Recently, the solution structure of Bcl-2 has been determined by NMR spectroscopy (Petros *et al.* 2001). This structure is similar to that previously obtained of Bcl-xL and resembles that of diphtheria toxin, a protein that kills cells by punching holes in their membranes. Indeed, some Bcl-2 family members such as Bcl-2, Bcl-xL, and Bax, can form ion channels in synthetic lipid membranes (Schlesinger *et al.* 1997). It has been reported that apoptosis-triggering Bcl-2 family members release cytochrome c and other proteins such as AIF (apoptosis-inducing factor) or Smac/DIABLO from the mitochondria by creating pores in the mitochondrial outer membrane, while antiapoptotic members somehow preserve the mitochondrial integrity (Desagher and Martinou 2000, Ferri and Kroemer 2001). Pro-apoptotic Bcl-2 proteins have been implicated in the opening of the mitochondrial permeability transition (PT) pore (Marzo *et al.* 1998) allowing the release of apoptogenic factors from mitochondria into the cytosol. These factors subsequently activate downstream executional phases, including the activation of death proteases, caspases. In addition, there have been several reports implying the role of Bcl-2 anti-apoptotic proteins in preserving cellular ATP levels and thus preventing cell death or switching necrosis to apoptosis (for review see Plas and Thompson 2002). Taken together, all these observations point to an intimate link between mitochondrial physiology and apoptosis, particularly the Bcl-2 family of proteins.

We have recently reported that Bcl-2 expression by vaccinia virus causes apoptosis in BSC-40 cells and several other cell lines, while it prevents apoptosis in HeLa G cells (Kalbáčová *et al.* 2002). We speculated that this dichotomy is caused by a different metabolic background of the two cell lines. To address this issue we focused on characterization of these cell lines with respect to their type of ATP synthesis. More importantly, we attempted to characterize the effect of Bcl-2 expression on mitochondrial respiration and on cellular ATP and ADP levels.

Methods

Materials

All media and growth supplements were purchased from Gibco BRL Life Technologies (Paisley, Scotland), PAA Laboratories GmbH (Linz, Austria), or Sigma Chemical Co. (St. Louis, MO), unless specified otherwise. Digitonin was purchased from Fluka (Buchs, Switzerland). Other chemicals were purchased from Sigma, unless specified otherwise.

Cells

African green monkey kidney-derived BSC-40, and human cervical carcinoma HeLa G cell lines were grown in Dulbecco's modified Eagle medium (DMEM; glucose 4.5 g/l) supplemented with 10 % heat-inactivated neonatal calf serum (NCS; 10 % NCS-DMEM). All media included penicillin (1 x 10^5 U/l) and streptomycin (100 mg/l). Cells were maintained at 37 °C, in a 5 % CO_2 atmosphere with 95 % humidity.

Recombinant viruses, virus growth, and titration

Recombinant vaccinia viruses (VV) expressing Bcl-2 (WRBcl2) or control chloramphenicol acetyltransferase (CAT; WRCAT) under the control of VV late promoter p4b were generated and kindly provided by Dr. S. B. Lee (Lee *et al.* 1993). Expression of the recombinant gene in these viruses is repressed by LacI and induced by IPTG. The viruses were propagated in BSC-40 cells supplemented with 2 % NCS-DMEM and crude stocks of viruses were prepared (Joklik 1962). Virus titers were determined by serial dilutions and plaque assays in BSC-40 cells. For the experiments, virus inocula were added in serum-free media at multiplicity of infection 2, and allowed to adsorb for 1 h in the presence of 1.5 mM IPTG. After removal of virus inocula, cells were once washed with the medium, and supplemented with the medium containing 2 % NCS and 1.5 mM IPTG. At indicated times after infection (h.p.i. – hours post infection), the cells were collected and processed as described below. For virus titrations, the cells were resuspended directly in the culture medium, lysed by two cycles of freezing-thawing and sonication, and virus yields were determined by serial dilutions and plaque assays in BSC-40 cells.

ATP and ADP determination

Total levels of ATP and ADP in the samples were detected in perchloric acid (PCA) extracts of the infected cells (Stratford and Dennis 1994). At each time point, the cells were collected in a culture medium by pipetting, washed twice with PBS and resuspended in 120 µl of 5 % PCA. After 5 min incubation on ice and a short centrifugation, 100 µl of the supernatant were neutralized with 22.5 µl of 4 M KOH, 0.4 M K_2HPO_4. Concentrations of ATP and ADP in cell extracts were determined by high-performance liquid chromatography (HPLC) on reversed-phase column (Separon SGX C18, 5 µm, 3 x 150 mm; Tessek, Czech Republic) using a modification of the previously published method (Micheli *et al.* 1993). The buffer used for isocratic elution

consisted of 0.1 M potassium phosphate, pH=7, methanol (85:15, v:v) and 8 mM tetrabutylammonium hydrogen sulfate. The flow rate was 0.6 ml/min. Five µl of cell extracts were injected onto the column, and the UV absorption was monitored at 260 nm. The retention times of ADP and ATP were 5.6 and 6.8 min, respectively. Quantification was carried out using calibration curves obtained by repeated injections of standard solutions of ADP and ATP at three different concentrations.

Cytochrome c oxidase activity assay

Cytochrome c oxidase (COX; EC 1.9.3.1) activity was measured essentially as described (Wharton and Tzagoloff 1967). Briefly, the rate of oxidation of reduced cytochrome c (50 µM final concentration; reduced with sodium dithionite, desalted on Sephadex G-25 column) was followed spectrophotometrically at 550 nm. All measurements were done using 0.5 x 10^6 of digitonized cells in a 10 mM potassium phosphate buffer (K_2HPO_4 and KH_2PO_4, pH 7.4) at 30 °C. The initial rate of cytochrome c oxidase in the sample was expressed as nmol/min/10^6 cells.

Polarographic measurement of respiration

Oxygen consumption was measured by the high-resolution respirometer – Oxygraph (Oroboros; Innsbruck, Austria), which enables sensitive measurements of oxygen kinetics at low oxygen partial pressure (Gnaiger *et al.* 1995). Cells (5 x 10^6/ml) were measured either permeabilized by digitonin (optimal concentration of 0.02 mg/1x10^6 cells determined by trypan blue staining; freshly prepared) in a KCl medium (80 mM KCl, 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM KH_2PO_4) or non-permeabilized in the cultivation medium. Oxygen consumption was monitored at 30 °C and expressed as pmol oxygen.s^{-1}.10^{-6} cells. The following respiratory substrates and inhibitors were added during the measurement: pyruvate (final concentration 5 mM) + malate (1.5 mM), ADP (1.5 mM), rotenone (1.5 µM), succinate (10 mM), oligomycin (5 µM), FCCP (1.5 µM) and antimycin A (1.5 µM). The signals from the oxygen electrode were recorded at 1-s intervals on a computer-driven data acquisition system (Datlab software, Oroboros, Innsbruck, Austria).

Table 1. Oxygen consumption by permeabilized cells in KCl medium.

	Oxygen consumption (pmol.s^{-1}.10^{-6} cells)		
	HeLa G cells	**BSC-40 cells**	**(% of HeLa G)**
Pyruvate + Malate +ADP	20.6 ± 3.3	26.7 ± 2.5	130 %
Succinate + ADP	14.3 ± 2.4	30.5 ± 5.3	213 %
FCCP	15.5 ± 2.8	35.3 ± 5.8	228 %

Oxygen consumption by cells permeabilized with digitonin was measured in a KCl medium as described in Materials and Methods. Results represent means of three (n = 3) independent experiments ± S.E.M.

Results

Energy metabolism of BSC-40 and HeLa G cells

In order to characterize the bioenergetical properties of cells used in this study, we measured the respiration of BSC-40 and HeLa G cells. Respirometry curves of permeabilized cells in the presence of various respiratory substrates and inhibitors are shown in Figure 1, and the corresponding values of oxygen consumption are summarized in Table 1. Oxygen consumption using pyruvate and malate as substrates (Complex I) by BSC-40 cells was 130 % of that by HeLa G cells. In addition, the respiration was stimulated by addition of ADP (state 3 respiration) to approximately 650 % in BSC-40 cells and to about 240 % in HeLa G cells, indicating a coupled state of mitochondria in both cell lines. The respiration with succinate as a substrate that reflects activity of the flavoprotein-dependent succinate dehydrogenase (Complex II) was approximately twice as high (213 %) in BSC-40 as in HeLa G cells. Finally, an uncoupler FCCP was used to assess the maximal capacity of the mitochondrial respiratory chain. Again, oxygen consumption by BSC-40 cells was roughly twice as high (228 %) as that by HeLa G cells. Figure 1 clearly shows that respiration with pyruvate and malate, and the respiration supported by succinate were almost 100 %

sensitive to rotenone, and antimycin A, respectively. Oligomycin (specific inhibitor of ATP synthase) caused the inhibition of ADP-stimulated respiration.

Additionally, we determined the activity of cytochrome c oxidase – the last and key enzyme complex of the respiratory chain (complex IV) – in permeabilized BSC-40 and HeLa G cells. The spectrophotometrically observed values were 32.0±1.6 and 19.7±0.2 nmol. $min^{-1}.10^{-6}$ cells (mean ± S.E.M., n=3), respectively. We also examined cytochrome c oxidase-dependent oxygen consumption in the presence of artificial substrates ascorbate and TMPD. The respiration was not increased by the addition of cytochrome c, indicating that the outer mitochondrial membrane in digitonin-permeabilized cells remained intact (data not shown).

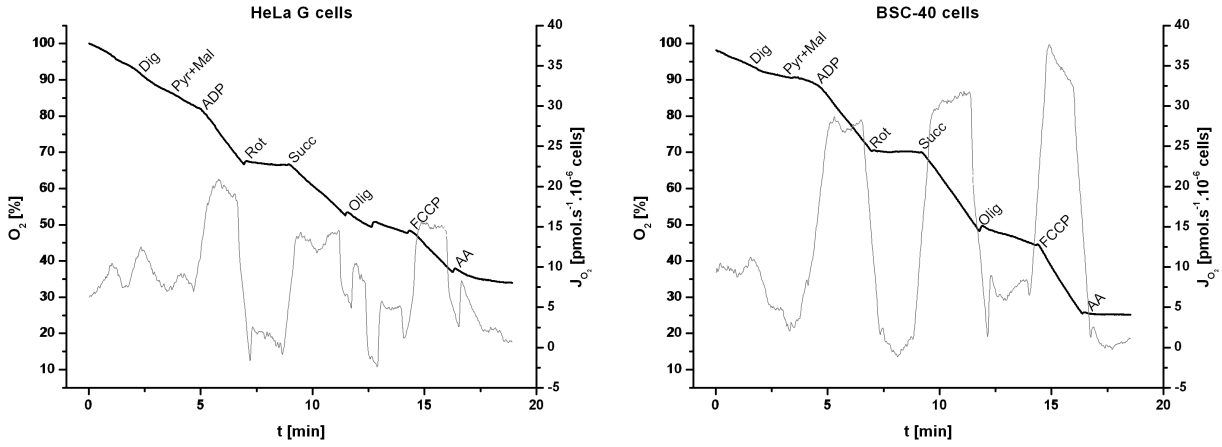

Fig. 1. *Oxygen consumption by permeabilized cells in KCl medium. Oxygen consumption by cells permeabilized with digitonin was measured in KCl medium as described in Materials and Methods. Bold line shows the percentage of initial oxygen tension O_2. Thin line represents flux of oxygen J_{O2} (pmol.s^{-1}.10^{-6} cells). Abbreviations denote chemicals added into the Oxygraph chamber (Dig – Digitonin, Pyr + Mal – Pyruvate + Malate, Rot – Rotenone, Succ – Succinate, Olig – Oligomycin, AA – Antimycin A) in concentrations specified in Materials and Methods.*

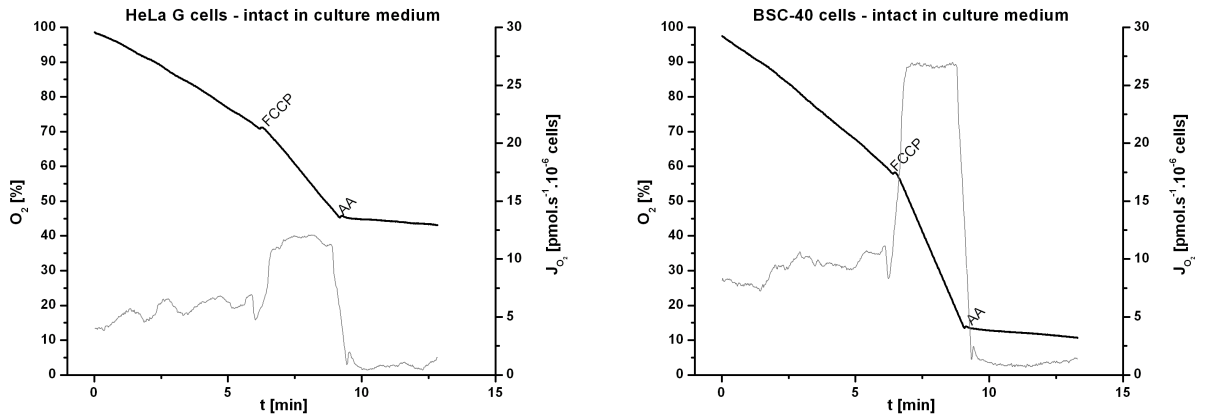

Fig. 2. *Oxygen consumption by intact cells in culture medium. Oxygen consumption by intact cells was measured in culture medium as described in Materials and Methods. AA – Antimycin A. For description of the graphs see Figure 1.*

Table 2. Oxygen consumption by intact cells in culture medium.

	Oxygen consumption (pmol.s-1.10^{-6} cells)		
	HeLa G cells	**BSC-40 cells**	**(% of HeLa G)**
Basal respiration	5.9 ± 1.4	9.3 ± 2.5	158 %
FCCP	10.2 ± 1.8	28.3 ± 4.6	277 %

Oxygen consumption by intact cells was measured in culture medium as described in Materials and Methods. Results represent mean of three (n = 3) independent experiments ± S.E.M.

Table 3. Oxygen consumption by permeabilized infected cells - BSC-40 cells (A), HeLa G cells (B).

A) BSC-40 cells

Time (h.p.i.)	**Infection**	**Oxygen consumption (nmol/10^6 cells)**			
		Pyruvate + Malate + ADP		**Succinate + ADP**	
			Bcl-2/CAT (%)		**Bcl-2/CAT (%)**
12	CAT	23.1 ± 3.9	87 %	36.4 ± 5.4	82 %
	Bcl-2	20.0 ± 4.7		29.8 ± 5.7	
15	CAT	21.8 ± 3.4	79 %	26.6 ± 3.7	71 %
	Bcl-2	17.2 ± 2.2		18.8 ± 3.7	
18	CAT	17.0 ± 3.0	49 %	22.8 ± 3.2	76 %
	Bcl-2	8.3 ± 2.4		17.4 ± 2.8	

B) HeLa G cells

Time (h.p.i.)	**Infection**	**Oxygen consumption (nmol/10^6 cells)**			
		Pyruvate + Malate + ADP		**Succinate + ADP**	
			Bcl-2/CAT (%)		**Bcl-2/CAT (%)**
24	CAT	15.7 ± 1.9	75 %	10.3 ± 2.6	83 %
	Bcl-2	11.7 ± 3.2		8.5 ± 2.4	
48	CAT	9.1 ± 2.2	56 %	13.1 ± 1.3	62 %
	Bcl-2	5.1 ± 0.6		8.1 ± 1.4	

Cells were infected with 2 PFU/cell of WRBcl2 (Bcl-2) or control WRCAT (CAT) in the presence of IPTG. At indicated h.p.i. (hours post infection), cells were collected, and the respiration was measured after permeabilization with digitonin in KCl medium as described in Materials and Methods. Results represent mean of three (n = 3) independent experiments ± S.E.M.

In addition to measuring oxygen consumption by permeabilized cells, we determined oxygen consumption by intact, non-permeabilized cells in a culture medium. This condition reflects the *in vivo* situation but prevents the use of some substrates and inhibitors due to their polarity and inability to cross the plasma membrane. These results are shown in Figure 2 and Table 2. Basal respiration (oxygen consumption by cells without any exogenously added substrate or inhibitor) as well as the respiration stimulated by the uncoupler FCCP were both

higher in BSC-40 cells than in HeLa G cells (158 % and 277 %, respectively).

To study the metabolism of BSC-40 and HeLa G cells further, we determined the concentrations of total cellular ADP and ATP nucleotides in both cell lines. Total cellular ATP levels were found to be lower in untreated HeLa G than in BSC-40 cells (5.20 and 9.47 nmol.10^{-6} cells, respectively; see Time 0 in Table 4). Likewise, the calculated ATP/ADP ratio determined in uninfected cells was lower in HeLa G than in BSC-40 cells (3.2 and 8.8, respectively; see Time 0 in Table 4). These results together with the measurements of oxygen consumption described above indicate that BSC-40 cells derive their ATP mainly from oxidative phosphorylation whereas HeLa G cells from glycolysis.

Bcl-2 inhibits the mitochondrial respiration of BSC-40 and HeLa G cells

After the characterization of uninfected cells, we assessed the effect of vaccinia virus-driven expression of Bcl-2 on cellular metabolism of BSC-40 and HeLa G cells. The time points analyzed in BSC-40 and HeLa G cells were chosen after a thorough analysis of the kinetics of VV-driven protein expression and morphological changes of these cells (Kalbáčová *et al.* 2002).

Table 4. Bcl-2-mediated changes of cellular ATP and ADP levels in BSC-40 cells (A) and HeLa G cells (B).

A) BSC-40 cells

Time (h.p.i.)	Infection	ATP (nmol/10^6 cells) ± S.E.M.	ADP	ATP/ADP ratio	Bcl-2/CAT (%)
0	0	9.47 ± 0.98	1.08 ± 0.23	8.8	
6	CAT	9.95 ± 1.29*	1.31 ± 0.37*	7.6	95 %
	Bcl-2	9.77 ± 1.52*	1.35 ± 0.34*	7.2	
12	CAT	9.15 ± 0.79	1.37 ± 0.19	6.7	91 %
	Bcl-2	8.49 ± 1.40	1.39 ± 0.15	6.1	
18	CAT	8.87 ± 0.30	1.60 ± 0.06	5.5	67 %
	Bcl-2	5.79 ± 0.65	1.58 ± 0.07	3.7	
24	CAT	8.29 ± 0.18	1.46 ± 0.06	5.7	60 %
	Bcl-2	4.55 ± 0.51	1.32± 0.10	3.4	

B) HeLa G cells

Time (h.p.i.)	Infection	ATP (nmol/10^6 cells) ± S.E.M.	ADP	ATP/ADP ratio	Bcl-2/CAT (%)
0	0	5.20 ± 0.74	1.64 ± 0.25	3.2	
24	CAT	3.12 ± 0.17	1.14 ± 0.07	2.7	96 %
	Bcl-2	3.64 ± 0.50	1.41 ± 0.05	2.6	
36	CAT	2.40 ± 0.18	1.03 ± 0.07	2.3	91 %
	Bcl-2	3.76 ± 0.29	1.83 ± 0.09	2.1	
48	CAT	1.98 ± 0.32	0.84 ± 0.10	2.4	70 %
	Bcl-2	4.28 ± 0.37	2.27 ± 0.10	1.9	

*Cells were infected with 2 PFU/cell of WRBcl2 (Bcl-2) or control WRCAT (CAT) in the presence of IPTG. At indicated h.p.i. (hours post infection), cells were collected, and the levels of ATP and ADP were determined in the perchloric acid extracts using HPLC as described in Materials and Methods. Results represent mean of three (n = 3) or two (*n = 2) independent experiments performed in duplicates ± S.E.M.*

Table 3A summarizes results obtained for permeabilized BSC-40 cells infected with vaccinia virus recombinants expressing either Bcl-2 oncoprotein (WRBcl2) or control CAT (chloramphenicol acetyl-transferase; WRCAT) at 12, 15 and 18 h.p.i. These data show a time-dependent decrease of pyruvate + malate- (Complex I), and succinate-supported (Complex II) respiration in WRBcl2-infected cells compared to those infected with control WRCAT, which exhibit a smaller decrease. Similar decrease can also be observed for the basal respiration (data not shown). Results analogous to those in permeabilized infected cells were also obtained in intact infected cells in culture medium (data not shown). As presented for HeLa G cells in Table 3B, expression of Bcl-2 leads to a decrease of respiration in these cells in a way similar to BSC-40 cells. Again, the study of oxygen consumption by intact infected cells in culture medium revealed a comparable decrease of respiration (data not shown). In both BSC-40 and to a lesser extent in HeLa G cell lines, the Bcl-2-caused inhibition of respiration was more pronounced with NADH-dependent substrates (pyruvate + malate).

Bcl-2-mediated effects on cellular ATP and ADP levels

Again, as in the case of uninfected cells, we determined total cellular ATP and ADP levels during the course of infection with vaccinia virus recombinants expressing Bcl-2 or CAT. As shown in Table 4, a time-dependent decrease of ATP/ADP ratio can be observed upon expression of Bcl-2 in both BSC-40 and HeLa G cells. In BSC-40 cells (Table 4A), the decrease of ATP/ADP ratio was more pronounced than in HeLa G cells (Table 4B), that revealed a low ratio of ATP/ADP concentration already at time 0.

Discussion

In the first part of our study, we characterized the energy metabolism of cells used for our experiments. By measuring the oxygen consumption, as well as by determining the cytochrome c oxidase activity, that allowed us to measure the maximal capacity of the respiratory chain and thus to complement results obtained from oxygen consumption measurements in the presence of an uncoupler FCCP, we found higher values for BSC-40 than for HeLa G cells. Additionally, an important indicator of the type of cellular metabolism – the ratio of concentrations of ATP and ADP nucleotides – was higher in BSC-40 than in HeLa G cells. All these observations taken together indicate that BSC-40 cells derive their ATP mainly from oxidative phosphorylation, whereas HeLa G cells derive ATP mostly from glycolysis. Indeed, it has been previously demonstrated that HeLa G cells depend primarily on glycolysis (Eguchi *et al.* 1997). This finding is perhaps not unexpected since HeLa G cells are of cancer origin (human cervical carcinoma) and it is known that cancer cells derive their energy mainly from glycolysis (Pedersen 1978).

To determine the effect of Bcl-2 expression on cellular metabolism we measured oxygen consumption by cells infected with vaccinia virus recombinants expressing Bcl-2 oncoprotein (WRBcl-2) or CAT control protein (WRCAT). Infection by the control VV recombinants with CAT allowed us to determine the effect of vaccinia virus infection on cells. Compared to the effect of VV itself (WRCAT), Bcl-2 expression caused a more profound, time-dependent decrease of pyruvate + malate- (Complex I), and succinate-supported (Complex II) respiration. In both BSC-40 and to a lesser extent in HeLa G cell lines, the Bcl-2-caused inhibition of respiration was more pronounced in the case of NADH-dependent substrates (pyruvate + malate) which might suggest higher susceptibility of Complex I to Bcl-2-mediated inhibition, supporting recent observations by Schwarz *et al.* (2001).

Finally, we determined total cellular ATP and ADP levels in the cells during the course of infection with vaccinia virus recombinants expressing Bcl-2 or CAT. In both BSC-40 and HeLa G cells, Bcl-2 caused a time-dependent decrease of ATP/ADP ratio. In BSC-40 cells (Table 4A), the levels of ATP as well as ADP remained comparable in cells infected by VV expressing Bcl-2 or control CAT until 12 h.p.i., the time when Bcl-2-induced morphological changes typical for apoptosis can be observed (Kalbáčová et al. 2002). At 18 h.p.i., Bcl-2 induced a decrease of ATP levels, while ADP levels remained unchanged, lowering the ratio of ATP/ADP concentrations. Thus it seems that either the decrease of ATP/ADP ratio or a decrease of ATP concentration itself might be responsible for death of BSC-40 cells. Alternatively, decrease of ATP might be secondary to another Bcl-2-mediated event leading to death of BSC-40 cells.

In contrast, expression of Bcl-2 in HeLa G cells (Table 4B) induced a gradual increase of ADP levels that was not accompanied by any major changes in ATP

levels. In this case Bcl-2 seemed to stabilize ATP levels that were otherwise decreased upon infection of HeLa G cells with control WRCAT. Thus, this relatively stable level of ATP might promote survival of these cells; alternatively, HeLa G cells survival might be supported by fairly constant ratio of ATP/ADP concentrations. The sum of ATP and ADP nucleotide concentrations decreases in HeLa G cells infected with WRCAT, while the expression of Bcl-2 keeps this sum rather unchanged. Such a decrease might reflect the protective role of Bcl-2 against nucleotide depletion caused by vaccinia virus infection. As we did not measure the concentration of AMP, we cannot conclude whether the depletion of ATP and ADP is accounted for by the increase of AMP. BSC-40 cells exhibit a decrease of the sum of ATP and ADP concentrations upon infection by WRBcl-2 reflecting the pro-apoptotic effect of Bcl-2.

Our results demonstrate that Bcl-2 affects mitochondrial energetics, in particular by modulating activity of Complex I. Whether this inhibition is mediated by direct interaction of Bcl-2 with components of the mitochondrial respiratory chain or possibly with voltage-dependent anion channel (VDAC) and/or mitochondrial ATP-ADP exchange through adenine nucleotide translocator (ANT) (reviewed by Plas and Thompson 2002, Tsujimoto and Shimizu 2002) remains to be elucidated.

Given the unexpected pro-apoptotic effects of Bcl-2 expression in BSC-40 and several other cell lines (Kalbáčová *et al.* 2002), and its well-known anti-apoptotic effects as shown in HeLa G cells (Diaz-Guerra *et al.* 1997, Lee *et al.* 1997, Mělková *et al.* 1997) one should consider the role of cellular background in different cell lines. These cell line differences might be eliminated by using one cell line in which the type of metabolism (mitochondrial respiration vs. glycolysis) was manipulated. These experiments are currently under way in our laboratory.

In conclusion, the results from respiration measurements as well as from the ATP/ADP determination indicate that BSC-40 cells derive their ATP mainly from oxidative phosphorylation whereas HeLa G cells depend on glycolysis. In both cell lines, Bcl-2 inhibits mitochondrial respiration and causes decrease of the ATP/ADP ratio over time. Given the ability of HeLa G cells to derive their energy mainly from glycolysis, and given the corresponding low ratio of ATP/ADP in these cells, it is conceivable to speculate, that Bcl-2 does not kill HeLa G cells as they are still able to keep the ATP levels and/or the ATP/ADP ratio fairly constant and thus to meet their energy needs. On the other hand, BSC-40 cells cannot cope with increased demand for energy equivalents by keeping ATP levels and/or the ATP/ADP ratio high enough, and consequently die. Based on these and on our previous results, we propose that the outcome of Bcl-2 oncoprotein expression is determined by the type of cellular ATP production, namely that Bcl-2 causes apoptosis in cells relying on oxidative phosphorylation.

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