Evidence against the hypothesis that BCL-2 inhibits apoptosis through an anti-oxidant effect

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Abstract

We contrasted possible protection against apoptosis afforded by either BCL-2 expression or anti-oxidant inhibitors in the same tumor target challenged by two distinct triggers of apoptosis. Exposure of L929 fibroblasts to tumor necrosis factor (TNF) or etoposide (VP-16) induced apoptotic death with similar kinetics. Enforced expression of BCL-2 significantly protected against apoptosis induced by VP-16 but had no effect against TNF-induced apoptosis. In contrast, the anti-oxidants desferrioxamine, butylated hydroxyanisol and N-acetyl cysteine all inhibited TNF-induced apoptosis in a concentration-dependent fashion. Although exposure to VP-16 resulted in a significant generation of intracellular oxyradicals, the above three anti-oxidant inhibitors had no effect on VP-16-induced apoptotic death. Interestingly, enforced expression of BCL-2 also inhibited the ability of VP-16 to generate oxy-radicals and to depress intracellular glutathione levels. These results indicate that BCL-2 can exert anti-oxidant effects but argue against the hypothesis that these effects are critical to its protection against apoptosis.

Keywords: anti-oxidants, TNF, BCL-2, VP-16

Abbreviations: TNF, tumor necrosis factor; ROI, reactive oxidant intermediate; BHA, butylated hydroxyanisol; NAC, N-acetyl cysteine; DCF, dichloro fluorescence; MCB, monochlor-obimane; PLA_2, phospholipase A_2

Introduction

Expression of the BCL-2 oncoprotein protects against apoptotic cell death induced by many different types of stimuli. The mechanism by which BCL-2 protects is still unclear. Two previous studies (Kane *et al*, 1993; Hockenbery *et al*, 1993) suggested that BCL-2's function as a survival factor was mediated through an anti-oxidant mechanism. This was based on the findings that BCL-2 expression can protect cells against oxidant stress and can inhibit the generation of reactive oxidant intermediates (ROIs) and/or their tissue destructive effects in conjunction with its anti-apoptotic influence (Kane *et al*, 1993; Hockenbery *et al*, 1993). An anti-oxidant role for BCL-2 would be consistent with a unifying hypothesis of apoptosis which alleges that intracellular reactive oxygen intermediates (ROIs) are common mediators of this death process (Buttke and Sandstrom, 1994). However, more recent data suggest BCL-2 can protect against non-oxidative injury. This is supported by the findings that BCL-2 protects against apoptosis occurring in very low oxygen tension where ROI generation is minimal (Shimizu *et al*, 1995; Jacobson and Raff, 1995). Furthermore, BCL-2 is known to protect against apoptotic death induced by other stimuli which are not known to stimulate the generation of ROIs, such as growth factor deprivation and c-*myc* expression (Nunez *et al*, 1990; Packham *et al*, 1996). One possible explanation for these divergent data is that BCL-2 blocks critical downstream events that are common to oxidative as well as non-oxidative triggers of apoptosis. However, if this were true, the ability of BCL-2 to inhibit intracellular ROI generation would be unexplained.

To further investigate this issue, we studied the effects of anti-oxidants and BCL-2 expression on tumor necrosis factor (TNF)- and etoposide (VP-16)-induced apoptosis as well as ROI generation and glutathione levels. TNF-induced cell death has been presented as supportive evidence of an important role for ROIs in apoptosis (Hennet *et al*, 1993; Yamauchi *et al*, 1989; Schulze-Osthoff *et al*, 1992; Wong *et al*, 1989). However, TNF also induces cell death by non-apoptotic pathways (Laster *et al*, 1988) and it is possible that the non-apoptotic death is the one that is mediated by ROIs. Furthermore, the effects of the putative anti-oxidant BCL-2 on TNF cytotoxicity are inconsistent (Vanhaese-broeck *et al*, 1993; Fernandez *et al*, 1995; Vandenabeele *et al*, 1995; Itoh *et al*, 1993). One possible explanation for these inconsistencies is that whether TNF exerts its cytotoxicity by apoptotic *versus* non-apoptotic or oxidative *versus* non-oxidative mechanisms may be target dependent and, thus, whether BCL-2 prevents cell death may also be target dependent. To avoid this potential problem, we studied the effects of anti-oxidants and enforced BCL-2 expression in the same target cell, the L929 fibroblast.

We also used VP-16 as an inducer of apoptosis in this model because it is thought to be sensitive to BCL-2 mediated protection (Kamesaki *et al*, 1993; Dole *et al*, 1994) and mediates death by non-oxidative pathways (Holm *et al*, 1989). Thus, at first glance, VP-16 seems a prototype for BCL-2-induced protection against non-oxidative injury. However, earlier literature indicates that VP-16 may generate intracellular ROIs (Mans *et al*, 1990;

Haim *et al*, 1986) and the role of these ROIs in VP-16 apoptosis has never been investigated. Our results show that TNF-induced apoptosis in this model is inhibited by antioxidants but not affected by BCL-2 expression. In contrast, VP-16-induced apoptosis of the same target is inhibited by BCL-2 but not by anti-oxidants. However, BCL-2 exerted anti-oxidant effects in VP-16-treated cells. These results suggest that BCL-2 can curtail ROI generation but argue against the hypothesis that these anti-oxidant effects are crucial to its protection against apoptosis.

Results

TNF and VP-16 induce apoptotic death in L929 target cells

As we have previously described (Fady *et al*, 1995), the death of L929 cells exposed to TNF is associated with the characteristic morphology of apoptosis and endonucleolytic cleavage of target cell DNA (Figure 1). These defining apoptotic changes were initiated approximately 8–12 h after treatment (Figure 1 and Mans *et al*, 1990), at a time when only minimal cytotoxicity was present ($26 \pm 3\%$ for 1.2×10^4 pM; $12 \pm 3\%$ for 6×10^3 pM at 12 h). They then increased over time to become maximal at 24 h of incubation. Death of the same target cells following incubation with VP-16 was associated with the same morphological abnormalities (not shown) and characteristic ladder-like DNA fragmentation (Figure 1). However,

apoptosis was related to VP-16 concentration as shown in Figure 1. It was consistently detected in cultures treated with concentrations of VP-16 > 200 μM. Although concentrations between 50 and 200 μM often resulted in a cytostatic effect, they never induced endonucleolytic cleavage of DNA. DNA ladder formation in VP-16-treated targets was also first detected at approximately 8–12 h of culture and was optimal by 24 h.

Enforced expression of BCL-2 protects L929 cells against VP-16 but has no effect on TNF-treated targets

L929 targets were transfected with the human BCL-2 gene or the empty vector (neo control) and then tested for sensitivity to TNF and VP-16. Efficient expression was achieved and confirmed by Western blotting. Transfected cells were not altered from parental cells in their morphology or doubling times (not shown). As shown in Figure 2, cytotoxicity induced by TNF after a 24 h exposure was unaffected by enhanced expression of BCL-2. When this assay was performed after only an 8 h exposure, the modest cytotoxicity of TNF (20% from 1.2×10^4 pM TNF) was similarly unaltered in BCL-2-expressing cells (18% cytotoxicity). In contrast, cytotoxicity induced by VP-16 used in concentrations below 400 μM was significantly curtailed in BCL-2-expressing targets (Figure 2). Protection was not detected in target cells challenged with VP-16 concentrations of 400 μM or
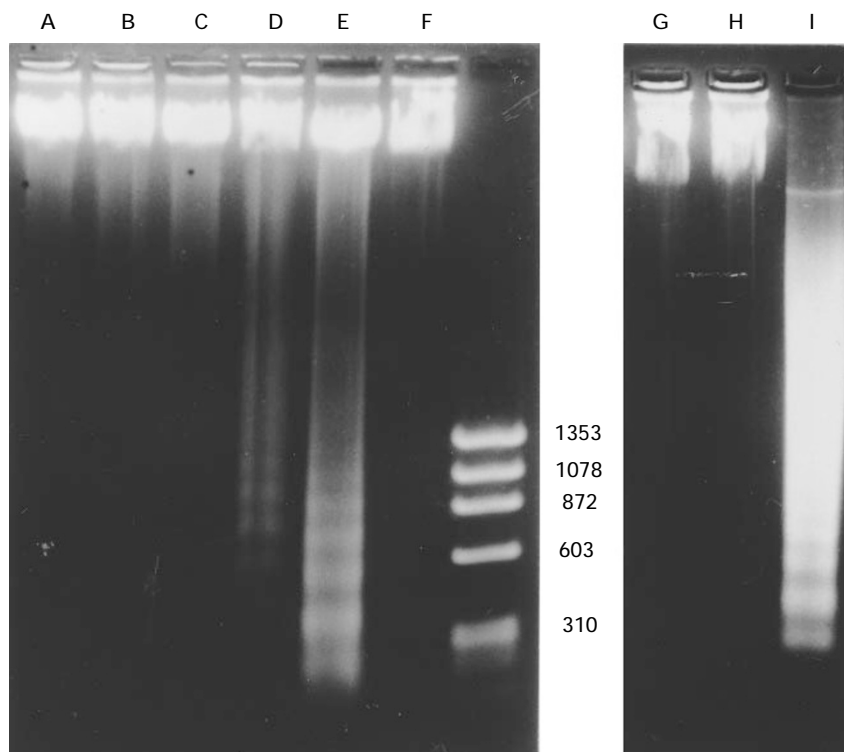

Figure 1 TNF and VP-16 induce endonucleosomal DNA fragmentation. DNA was isolated from L929 cells incubated with media for 24 h (control, lane F) or TNF (6×10^3 pM) for 4 (lane A), 6 (lane B), 8 (lane C), 12 (lane D) or 24 h (lane E) or VP-16 for 12 h at a concentration of 50 μM (lane G), 100 μM (lane H) or 250 μM (lane I). Locations in the gel of DNA size standards shown are indicated in base pairs. This experiment was repeated three times with identical results.

greater. Figure 3 demonstrates that BCL-2-mediated protection against 250 μM VP-16 was seen as a delay in cytotoxicity rather than a complete inhibiton. Morphologic examination of Giemsa-stained cytosmears confirmed that apoptotic changes caused by VP-16 was diminished in BCL-2 expressing cells. After 12 h incubation with VP-16, examination of neo control targets demonstrated 33% of the cells were apoptotic whereas only 15% of similarly treated BCL-2-expressing targets were apoptotic. Further evidence that the transfected BCL-2 gene was functional was demonstrated when the transfected L929 cells were found to be relatively resistant to apoptosis induced by low concentrations of hydrogen peroxide (Gardner *et al*, 1997). Figure 4 demonstrates that BCL-2-induced protection against VP-16 was associated with a decrease in endonucleosomal DNA fragmentation.
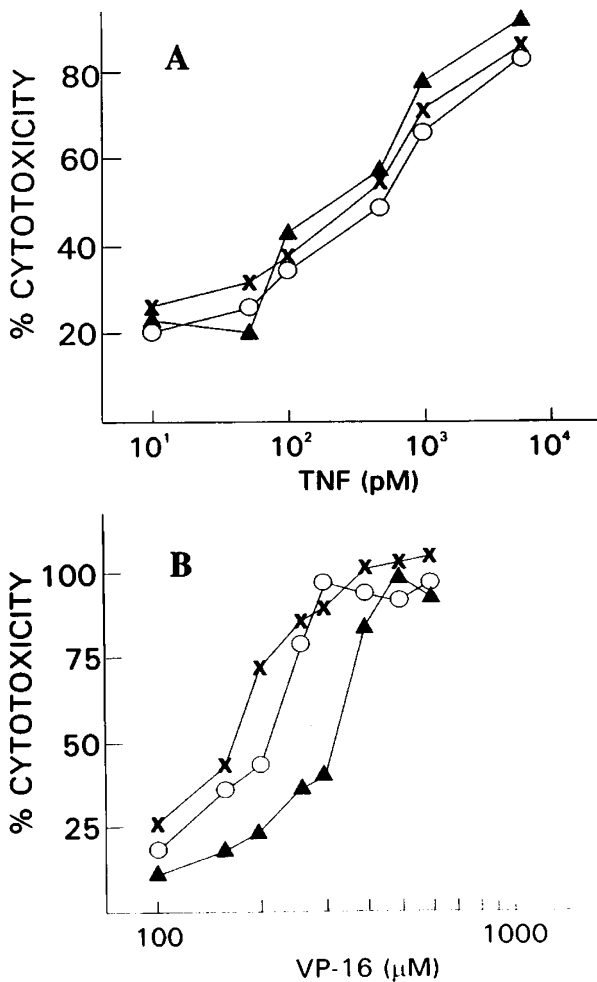
Antioxidants inhibit TNF-induced apoptosis but not VP-16-induced apoptosis in L929 cells

We utilized desferrioxamine, butylated hydroxyanisol (BHA) and N-acetyl cysteine (NAC) as anti-oxidants to test whether
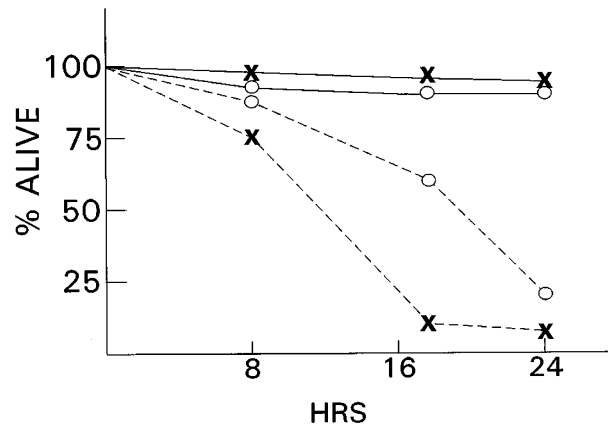

Figure 3 BCL-2 delays cytotoxicity induced by VP-16. Neo control targets cultured in media (X——X) or 250 μM VP-16 (X- - -X) for varying durations and compared to BCL-2-expressing transfected cells cultured in media (O——O) or 250 μM VP-16 (O- - -O). Viability determined by trypan blue exclusion. Results are means of quadruplicate samples. The SDs were <5% of the means. The experiment was repeated ×1 with identical results.
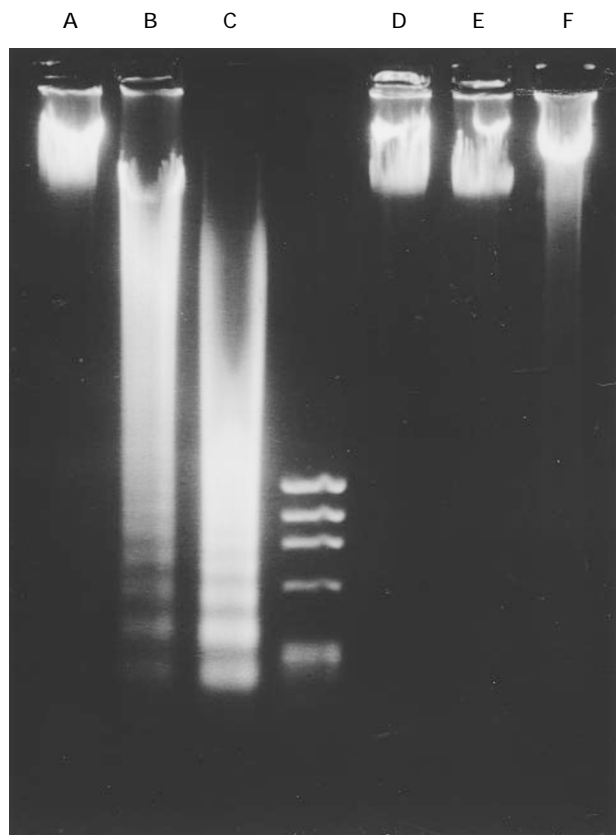

Figure 2 Effect of BCL-2 expression on TNF and VP-16 sensitivity. Parental L929 (O——O), neo control L929 (X- - -X) or BCL-2-expressing L929 cells (▲——▲) incubated with increasing concentrations of TNF for 24 h or VP-16 for 16 h and cytotoxicity assessed. Results are means of three separate experiments. The standard deviations (SDs) were <10% of the means in all cases. Cytotoxicity induced by TNF was not significantly different among the three targets. Cytotoxicity to BCL-2-expressing cells was significantly below that to parental and neo control cells for VP-16 concentrations of 250 and 300 μM.

Figure 4 BCL-2 inhibits VP-16-induced DNA fragmentation. DNA isolated from neo-control cells (**A**–**C**) or BCL-2-transfected cells (**D**–**F**) cultured in media for 24 h (lanes **A** and **D**) or VP-16 (300 μM) for 12 (lanes **B** and **E**) or 24 (lanes **C** and **F**) h.

TNF- or VP-16-induced apoptotic death was mediated by ROIs. These agents are unrelated and function as anti-oxidants through completely different mechanisms. Desferrioxamine is an iron chelator which suppresses hydroxyl radical formation by the Fenton reaction, BHA is a non-specific oxyradical scavenger and NAC, after uptake and deacylation, is converted to glutathione where it functions as a redox buffer and ROI scavenger. The anti-oxidant inhibitors were used in the highest concentrations possible that were not cytotoxic by themselves. Targets were exposed to desferrioxamine or NAC overnight prior to addition of VP-16 or TNF. Experiments with BHA were performed as co-cultures where BHA was added together with TNF or VP-16. All three anti-oxidant reagents significantly protected L929 targets against TNF-induced cytotoxicity in a concentration-dependent fashion (Figure 5). In general, BHA and NAC were more effective as inhibitors when compared to desferrioxamine. In addition, TNF-induced ladder-like DNA fragmentation was reduced by these agents (Figure 6). BHA and NAC were once again more effective with a complete prevention of any detectable DNA fragmentation while desferrioxamine only modestly inhibited ladder formation. In contrast to these results, these anti-oxidant inhibitors had no effect whatsoever on VP-16-induced cytotoxicity when used at the same concentrations. The ID50s for VP-16 in cultures exposed for 24 h were 270 μM in control VP-16-treated cells, 230–255 μM in cultures containing 50–200 μM of desferrioxamine, 250–280 μM in cells co-cultured with 10–200 μM BHA and 260–275 μM in cultures exposed to 25–50 μM of NAC. A similar lack of effect of these inhibitors on VP-16 cytotoxicity was seen when the assay was performed after only 8 h of incubation (not shown). As expected, the inhibitors also had no effect on VP-16-induced endonucleosomal fragmentation as well as morphologic changes characteristic of apoptosis (not shown).

VP-16 induces oxidant generation which is inhibited by anti-oxidants and BCL-2 expression

The DCF fluorescence assay was used to test whether VP-16 treatment induced the detectable generation of intracellular ROIs. L929 targets exposed to 250 μM VP-16 demonstrated a significant increase in DCF fluorescence which was time dependent (Figure 7). This was accompanied by a significant fall in the intracellular level of glutathione (Figure 8). The increase in DCF fluorescence was significantly inhibited by co-incubation with 200 μM of BHA (Figure 7), confirming the anti-oxidant's ability to inhibit ROI generation induced by VP-16. Interestingly, BCL-2 expression in transfected cells also curtailed the ability of VP-16 to induce ROI generation and deplete the level of cellular GSH (Figure 8).

We also attempted to use the DCF assay to monitor ROI generation in TNF-treated cells. However, we detected insignificant ROI generation in this assay (Figure 7). However, oxy-radical induced DNA injury was clearly detected by an ELISA assay utilizing an antibody (Zimmerman *et al*, 1989) specific for oxidant-mediated thymine glycol base modifications. The reactivity of the antibody with TNF-treated L929 DNA was specifically inhibited by pre-incubation with osmium tetroxide-treated L929 DNA but not by S1 endonuclease-treated L929 DNA.

As shown in Table 1, TNF treatment results in extremely rapid generation of DNA thymine glycols first detected as
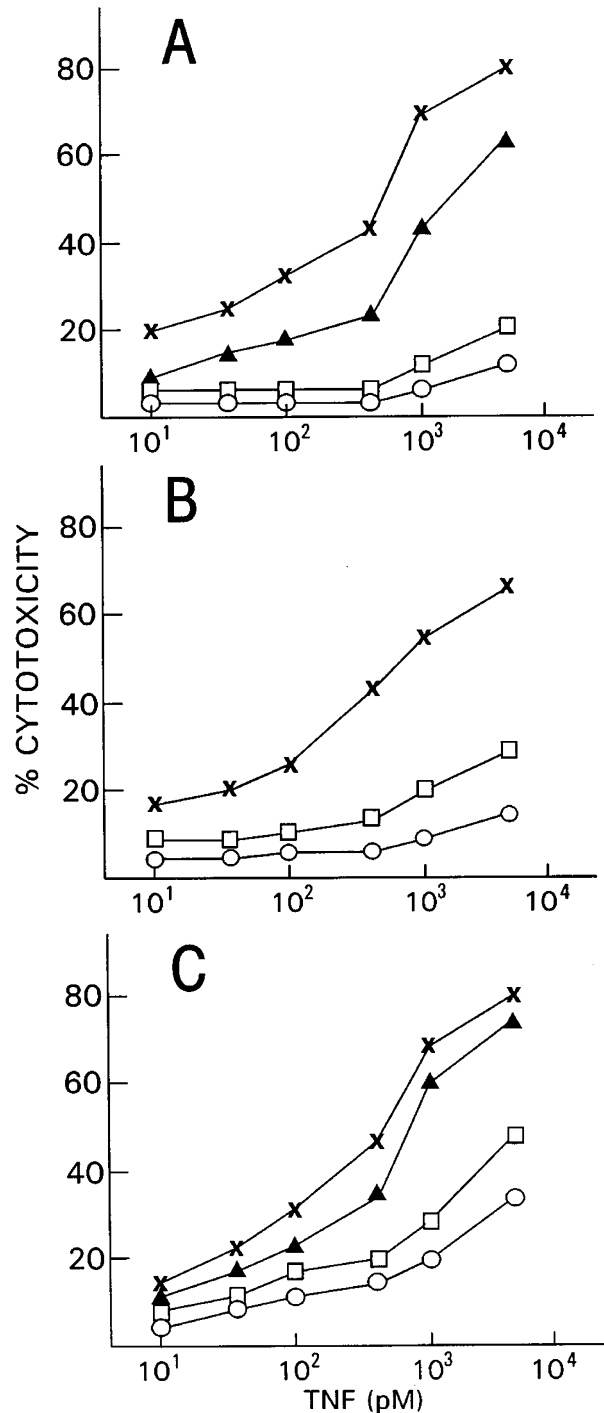

Figure 5 Anti-oxidants inhibit TNF cytotoxicity. L929 cells cultured for 24 h with increasing concentrations of TNF ($10^1 - 10^4$ pM) ± BHA in (**A**) (X——X), control, BHA=0; ○——○, BHA=100 μM; □——□, BHA=50 μM; ▲——▲, BHA=10 μM), NAC in (**B**) (X——X, control, NAC=0; ○——○, NAC=50 mM; □——□, NAC=25 mM) and desferrioxamine in (**C**) (X——X, control, des=0; ○——○, des=200 μM; □——□, des=100 μM; ▲——▲, des=50 μM). Cytotoxicity assessed by methylene blue assay. Results are means of four separate experiments for each inhibitor. SDs were < 10% of the means in all cases.
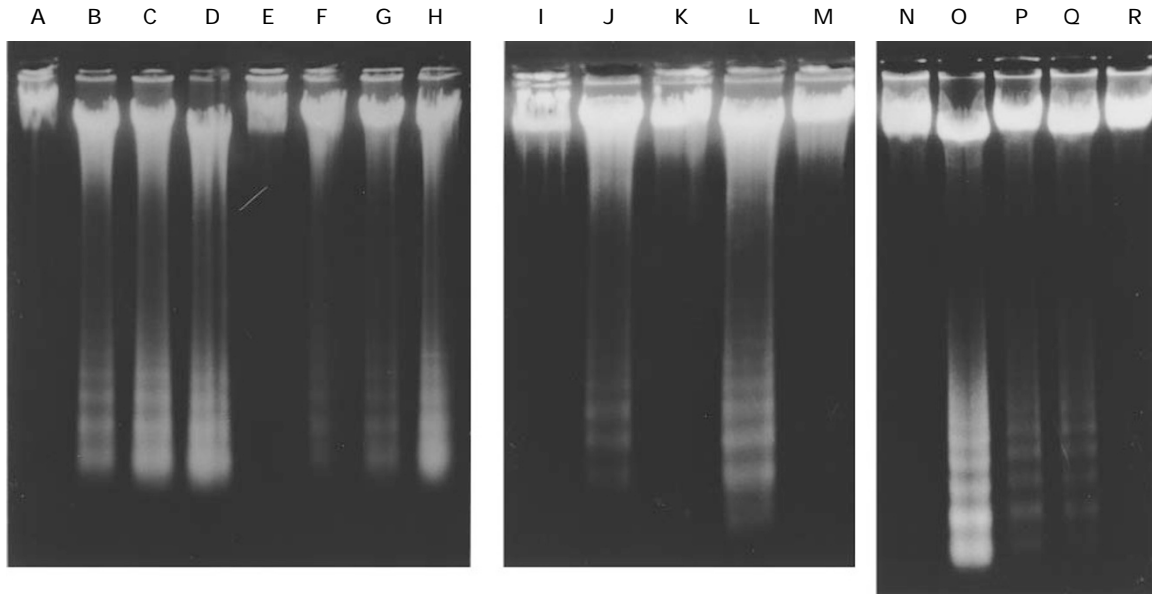
Figure 6 Effect of desferrioxamine (**A–H**), BHA (**I–M** and NAC (**N–R**) on TNF-induced DNA fragmentation. In the first panel (**A–H**) cells incubated in media alone (lane **A**), increasing concentrations of TNF alone (6×10^2, 1.2×10^3 and 6×10^3 pM, lanes **B**, **C** and **D** respectively), desferrioxamine alone (200 μM, lane **E**) or desferrioxamine+TNF (6×10^2, $1, 2 \times 10^3$ and 6×10^3 pM, lanes **F–H**). In the middle panel, cells incubated in media (lane **I**), TNF alone (6×10^2 and 6×10^3 pM, lanes **J** and **L**) or the combination of BHA (200 μM and TNF (6×10^2 and 6×10^3 pM, lanes **K** and **M**). In the third panel, cells incubated in media alone (lane **N**), TNF alone (6×10^3 and 6×10^2 pM, lanes **O** and **Q**) or the combination of NAC (50 mM) and TNF (6×10^3 and 6×10^2 pM, lanes **P** and **R**). All incubations for 20 h. All experiments repeated once with identical results.
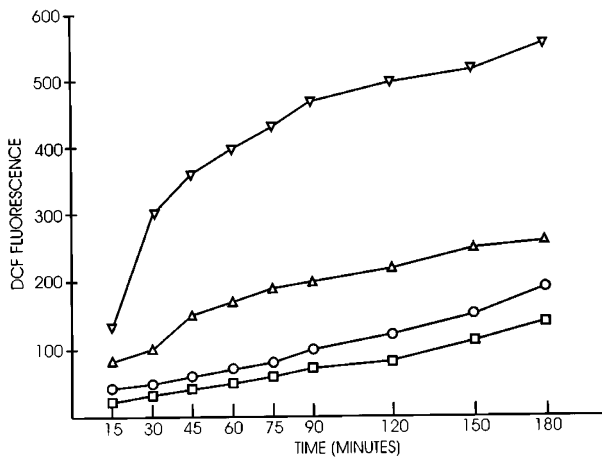

Figure 7 Intracellular generation of ROIs in L929 cells. DCF fluorescence measured over 180 minutes in L929 cells incubated alone ($\square$), in 6×10^3 pM TNF ($\bigcirc$), in 250 μM VP-16 ($\triangledown$) or 250 μM VP-16+200 μM BHA ($\triangle$). Fluorescence was normalized to cell numbers as determined in Materials and Methods. Each point represents the mean of triplicate wells. The standard errors were all <5% of the mean values. This experiment was repeated three times with similar results.
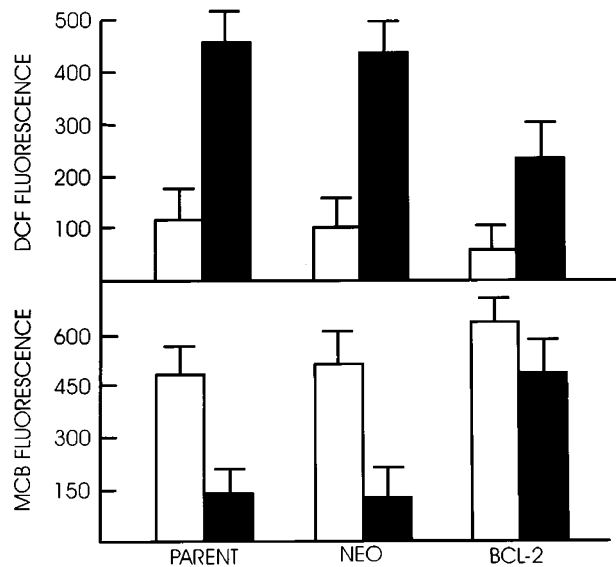

Figure 8 Effect of VP-16 on DCF fluorescence and GSH levels in BCL-2-transfected cells. Parent non-transfected, neo-control transfected (empty vector) or BCL-2-transfected L929 cells were assayed for ROI generation by DCF fluorescence (following 2 h incubation without (open bars) or with VP-16, 250 μM (darkened bars)) and for glutathione content by MCB fluorescence (open bars=no VP-16; darkened bars=VP-16, 250 μM). DCF and MCB fluorescence normalized for cell numbers. Values are means $\pm$ SD for three separate experiments. The increase in DCF fluorescence induced by VP-16 in BCL-2-transfected cells is significantly less than in parental or neo-control cells. The decrease in MCB fluorescence induced by VP-16 in BCL-2-transfected cells is significantly less than in parental or neo-control cells.

early as 2 h after treatment (in agreement with a previous report; Zimmerman *et al*, 1989). Desferrioxamine, BHA and NAC were all capable of preventing TNF-induced thymine glycol modification and the concentrations of these inhibitors that were effective correlated with the concentrations effective in protecting against TNF cytotoxicity (Table 2). A TNF-induced oxidative stress was also suggested by results of the GSH assay. TNF at 6×10^2 pM (for 2 h)

decreased the MCB fluorescence of L929 cells from 400 to 225 (mean of three separate experiments). Interestingly,

enforced BCL-2 expression in the transfected line had no effect on the ability of TNF to reduce GSH levels (MCB fluorescence decreased from 450 to 200 following a 2 h exposure to TNF).

Discussion

The results of the current study do not support the hypothesis that BCL-2 functions as an anti-oxidant to inhibit apoptosis. First, TNF induced apoptotic death of L929 targets, which was very sensitive to marked inhibition by co-incubation with anti-oxidant agents, was completely resistant to regulatory control by enforced expression of BCL-2. Second, VP-16-induced apoptotic death of the same targets was significantly delayed by BCL-2 expression but was completely resistant to the same anti-oxidant drugs that curtailed TNF apoptosis. Although VP-16 stimulated ROI generation and BCL-2-induced protection was associated with a diminished ROI generation, the fact that the anti-oxidant BHA, which depressed ROI generation equally (compare Figures 7 to 8), had no inhibitory effect on apoptosis, indicates that these alterations in oxidant production have no relevance to the degree of VP-16-induced cell death.

The results support previous investigations that also indicate BCL-2 can protect against non-oxidative injury. In studies by Shimizu *et al* (1995) and Jacobson and Raff (1995), BCL-2 was a successful protective influence in very low oxygen tensions when apoptosis was induced by staurosporine, anti-fas and growth factor depletion

(Jacobson and Raff, 1995) as well as hypoxia (Shimizu *et al*, 1995). Our results indicate that BCL-2-mediated protection against apoptosis induced by VP-16 is also independent of effects on ROIs. Our model of VP-16-induced apoptosis, however, was associated with concurrent ROI generation and glutathione depletion and, as such, allowed us to detect an anti-oxidant effect of BCL-2 which was not relevant to the oncoprotein's anti-apoptotic effect.

It is not clear from our data whether the anti-apoptotic and anti-oxidant effects of BCL-2 in our model are linked or dissociated. However, one possible unifying hypothesis is that BCL-2 suppresses apoptotic events that are upstream of ROI generation. Recent studies (Zamzami *et al*, 1996) suggest these events occur in mitochondria and represent opening of permeability transition (PT) pores, collapse of the mitochondrial transmembrane potential and release of a mitochondrial apoptotic protein into the cytosol. Furthermore, BCL-2 expression, with its specific localization to the outer mitochondrial membrane, prevents this sequence of events. Many different apoptotic signals appear to converge at the mitochondrial PT, including the inducers of apoptosis we used in the current study, TNF (Zamzami *et al*, 1996) and VP-16 (Shimizu *et al*, 1996). The VP-16-induced collapse of the mitochondrial transmembrane potential is likely mediated by p53 activation (Marchetti *et al*, 1996). Interestingly, hyper-production of ROIs occurs secondarily to the loss of mitochondrial transmembrane potential and uncoupling of mitochondrial respiration. Thus, we hypothesize that a VP-16-induced apoptotic signal stimulated opening of PT pores in L929 cells, release of an apoptotic mitochondrial protein which initiated an apoptotic cascade, and, only secondarily, hyperproduction of ROIs. Enforced expression of BCL-2 in L929 cells protected against apoptosis by preventing release of the apoptotic mitochondrial protein. As a secondary result, the hyperproduction of ROIs, which were not critical for apoptosis, was also down-regulated. This scenario could also explain the aforementioned results of Kane *et al* (1993) and Hockenbery *et al* (1993) where BCL-2 inhibited generation of peroxides and lipid peroxidation byproducts during protection against apoptosis.

In the current study, human TNF was used with murine fibroblast targets so that only the p55 receptor was engaged (Lewis *et al*, 1991). This engagement induced a cytotoxicity that was apoptotic in nature as endonucleoso-

Table 1 Generation of oxidative DNA damage in TNF-treated targets

TNF	Treatment duration	Thymine glycols/10^5 thymines
−	1 h	<1
−	2 h	<1
−	4 h	<1
−	6 h	<1
+	1 h	1.5
+	2 h	4.5
+	4 h	6.5
+	6 h	8

L929 cells incubated with (6×10^2) or without TNF for various intervals after which DNA was extracted and assayed for thymine glycol formation in an ELISA. This experiment was repeated three more times with similar results.

Table 2 Inhibition of TNF-induced cytotoxicity and thymine glycol formation by desferrioxamine (des), BHA and NAC

Drug	Concentration	TNF-induced cytotoxicity (pM)			TNF-induced thymine glycols/10^5 thymines		
		1.2×10^3	2.4×10^2	1.2×10^2	1.2×10^3	2.4×10^2	1.2×10^2
−	−	75%	63%	40%	9	6	4.5
des	200 μM	30*	18*	10*	2*	1*	1*
des	50 μM	68	59	34	7	5.5	2.5
BHA	100 μM	10*	5*	0*	1*	1*	1*
BHA	10 μM	58*	37*	22*	5*	3.5*	2*
NAC	50 mM	15*	9*	3*	1*	1*	1*
NAC	5 mM	59*	46	33	8	4.5	3

L929 cells exposed to TNF ($1.2 \times 10^2 - 1.2 \times 10^3$ pM) in presence or absence of desferrioxamine (des), BHA and NAC. Cytotoxicity assayed after 20 h of incubation and thymine glycol formation after 4 h. Data=values of three separate experiments. All standard deviations were less than 10% of the mean values. *Significantly different from control (no inhibitor, top line).

mal degradation as well as the characteristic morphological alterations were present and preceded detected cytotoxicity. Although we could not detect ROI generation in the DCF assay, an ELISA assay utilizing an antibody specific for oxy-radical induced DNA injury served as an indicator of ROI generation. In addition, GSH levels decreased in TNF-treated cells confirming the development of oxidative stress. Effective concentrations of the anti-oxidants that inhibited the generation of oxy-radical induced thymine glycols also inhibited TNF cytotoxicity and endonucleosomal fragmentation. Thus, we are confident that ROI generation plays an important role in TNF-induced apoptotic death within this model. The DCF assay may not be sufficiently sensitive for TNF-induced ROI detection; it is mostly sensitive to hydroperoxides rather than superoxide anion. O'Donnell *et al* (1995) was also unable to detect TNF-stimulated ROIs in L929 cells using the DCF assay while other studies, which utilized the dihydrorhodamine (Shoji *et al*, 1995) or hydroethidine oxidation (Zamzami *et al*, 1996) assays were more successful. Packham *et al* (1996) has also shown the DCF assay to be relatively insensitive to the generation of intracellular ROIs that accompany apoptosis when compared to these other assays.

Recent studies have further elucidated two new pathways by which TNF can induce apoptosis. In one, following ligand binding, the death domain of the p55 TNFR1 recruits the other death domain-containing proteins TRADD, FADD and RIP (Hsu *et al*, 1995, 1996) and, from this complex, the death effector domain of FADD recruits the ICE-like protease MACH/FLICE (Boldin *et al*, 1996; Muzio *et al*, 1996). Theoretically, MACH/FLICE becomes activated (through yet unknown mechanisms) and the apoptotic effector protease cascade is initiated. In the second, a novel second messenger, ceramide, is produced subsequent to ligand binding and sphingomyelinase activation (Kolesnik and Golde, 1994). When applied to cells, exogenous ceramide induces apoptosis (Obeid *et al*, 1993), providing support for its role in TNF-induced apoptosis. These two new potential pathways of apoptosis must be added to previously described mechanisms that are activated subsequent to ligand binding and which experimental data supports their involvement in TNF-induced apoptosis. These include the activation of other second messengers like arachidonic acid metabolites and intracellular calcium (reviewed by Beyart and Fiers, 1994) as well as the above mentioned effect on the mitochondrial PT. The multitude of possible TNF-induced pathways of apoptosis provides a possible explanation for the variable effects of BCL-2 (Vanhaesebroeck, Fernanadez, Vandenable, Itoh). Due to its subcellular localization, BCL-2 is well suited to block TNF apoptotic signals and ICE-like protease activation mediated via effects on the mitochondrial PT. However, if the protease cascade is initiated by MACH/FLICE when they are linked to p55 via FADD at the cell membrane or by a second messenger such as cytosolic phospholipase A_2 (PLA$_2$) (Voekel-Johnson *et al*, 1996), BCL-2 would be less effective. Although TNF may open PT in L929 cells, the lack of inhibitory effect of BCL-2 in our model indicates there must be other TNF-induced pathways in our cells, possibly related to protein recruitment to membrane p55 or PLA2 activation where ICE-like proteases are activated in a milieu protected from BCL-2.

It remains a challenge for investigators to integrate the newly discovered metabolic mechanism of TNF-induced apoptosis with its known oxygen or oxyradical dependence. The mechanism by which the anti-oxidant reagents we used inhibited cell death is still unclear but several possibilities can be considered. The first is that these anti-oxidants inhibited the generation of mitochondrial ROIs and, thus, curtailed apoptosis. Although the initial TNF-induced opening of mitochondrial PT only leads secondarily to hyperproduction of ROIs (Zamzami *et al*, 1995), increasing oxidant accumulation could provide a self-amplification loop as ROIs themselves can further alter PT and disrupt the mitochondrial transmembrane potential (Zamzami *et al*, 1995, 1996). This may be the mechanism by which low concentrations of hydrogen peroxide (Gardner *et al*, 1997) or other oxidants induce apoptosis and might explain why mitochondrial BCL-2 expression can protect against this injury. Thus, it is theoretically possible that inhibition of mitochondrial-generated ROIs with antioxidants could significantly decrease an apoptotic stimulus. We consider this unlikely since, as explained above, we would have expected BCL-2 expression to also inhibit apoptosis if it was initiated at the mitochondrial PT. Furthermore, BCL-2 expression did not alter the decrease in GSH induced by TNF, indicating this oxidative stress did not likely result from mitochondria. A second possibility is that TNF-induced ROIs that mediate apoptosis were generated in extra-mitochondrial sites. For example, cytosolic PLA$_2$ is necessary for apoptosis of melanoma cells induced by TNF plus metabolic inhibitors (Voekel-Johnson *et al*, 1996), TNF can activate PLA$_2$ in L929 cells (Hayakawa *et al*, 1993) and there is some support that ROI byproducts of the PLA$_2$-arachidonate pathway can mediate TNF cytotoxicity (Hayakawa *et al*, 1993; Matthews *et al*, 1987). A third possibility is that ROIs are not involved directly in TNF-induced apoptosis but that the redox state of the cell significantly regulates other apoptotic pathways. The intracellular redox state is buffered by thiol-type reducing molecules of which glutathione is the principal agent. The two most effective inhibitors in our study, NAC and BHA, would be expected to enhance this buffering capacity, NAC by its conversion to GSH following deacylation, and BHA, by its scavenging of ROIs and prevention of ROI-induced GSH oxidation and loss by the cell. In addition, an unrelated reducing agent, adult T-cell leukemia-derived factor (Matsuda *et al*, 1991), which also functions as a cytoplasmic reducing agent, protects against TNF. The redox potential might regulate inherently non-oxidative apoptotic pathways through its effect on signal-transducing protein tyrosine kinases and/or protein tyrosine phosphatases. For example, protein tyrosine phosphatases have a reactive cysteine residue at their catalytic sites which must be in the reduced form for activity (Staal *et al*, 1994). Further support for this theory comes from the finding that TNF stimulates extensive tyrosine phosphorylation in cells only if GSH has been depleted (Staal *et al*, 1994) and

protein kinase activity seems to be important for p55 signalling of apoptosis (Grell *et al*, 1994). Since alteration of the thiol buffering capacity would affect the cell's redox state by regulating the small amount of endogenous ROIs continuously produced even in the absence of an enhanced oxidative stress, this hypothesis could also explain several studies where antioxidant GSH/thiol-enhancing reagents can protect against apoptosis even in the absence of ROI production by the apoptotic signal in question (Packham *et al*, 1996; Hockenbery *et al*, 1993; Chiba *et al*, 1996; O'Donnell *et al*, 1995). Further support for this hypothesis comes from the effects of dithiothreitol and cycloheximide. The former sulfhydryl agent, which protects enzymes against redox-induced transition of sulfhydryl groups, protects against TNF cytotoxicity (Shoji *et al*, 1995). The latter inhibitor of protein synthesis significantly reduces levels of intracellular GSH (Chiba *et al*, 1996) and this reduced buffering capacity may play a role in the ability of cycloheximide to enhance TNF cytotoxicity.

In summary, the results of this study demonstrate that, although BCL-2 expression can decrease ROI generation within cells it is simultaneously protecting from apoptosis, its protective effects are not due to these anti-oxidant properties. The important role of ROIs in the distinct TNF-induced pathways that result in apoptosis remain to be clarified.

Materials and Methods

Target cell

The murine L929 fibroblast cell line was obtained from the American Type Culture Collection and maintained in RPMI medium supplemented with 10% fetal calf serum, glutamine and antibiotics.

Reagents

Recombinant human TNF was a gift from Genentech Inc. (South San Francisco, CA). The specific activity was 6.27×10^7 U/mg protein. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise designated.

Cytotoxicity assay

The methylene blue cytotoxicity assay was performed as previously described (Fady *et al*, 1995). Briefly, 10^4 cells in 100 μl were dispensed into a 96 well microtitre plate, allowed to adhere overnight and then exposed to serial dilutions of TNF or VP-16 in an additional 100 μl. After varying intervals, media and dead cells were removed, viable cells were fixed in 95% ethanol and then stained with 1% methylene blue. Following washing of plates, blue dye was solubilized in 100 μl HCl (1 M) and optical density read at 630 nm. Groups were run in quadruplicate and the standard deviation (SD) of replicates was always < 10% of the mean. Percent cytotoxicity was determined as $100 \times$ (ODcontrol − ODexp/ODcontrol). The concentration of TNF or VP-16 inducing 50% inhibition of cell survival (ID50) was extrapolated from results of the cytotoxicity assay.

Electrophoresis of DNA

As previously described (Fady *et al*, 1995) cells were lysed in 10 mM Tris-HCl (pH 7.5), 100 mM EDTA, 0.5% SDS and 100 μg/ml of proteinase K for 18 h at 37°. DNA was then extracted twice with phenol, PCI and chloroform, precipitated in ethanol, centrifuged (30 min at 10 000 g) and resuspended in TE buffer containing 100 μg/ml of RNase for 1 h at 37°. Following one more extraction in phenol, PCI and chloroform and precipitation on 70% ethanol, DNA was resuspended in TE buffer and 10 μg DNA per lane was electrophoresed in a 1% agarose gel for 2 h at 45 V. The gels were visualized with ethidium bromide.

BCL-2 transfection

The full length BCL-2 cDNA was a gift from Dr Dale Bredesen, UCLA. It was subcloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA) with EcoRI and XbaI sites. Plasmids for transfection were purified with Maxprep kit (Promega, Madison, WI). Transfection was performed according to manufacturer's instruction. Briefly, L929 cells were trypsinized and seeded at 10^6 cells per 100 mm dish 24 h prior to transfection. Plasmid and Dotap solution (Boehringer, Hague Road, IN) were diluted to 0.05 μg/ml and 1 μg/ml respectively with 200 mM HEPES buffer, pH 7.4. Diluted plasmid DNA and Dotap solution were mixed at a ratio of 1:2 and kept at room temperature for 15 min. One hundred and fifty μl of the above mixed solution was added to 10 ml of complete media for each sample. After mixing gently, it was used immediately to replace the culture media in each dish. After 10 h exposure, the transfection media was removed and cells were re-fed with fresh media for another 24 h. At that point, transfected cells were selected in G418 (GIBCO BRL, Grand Island, NY).

Assay for generation of intracellular ROIs

Intracellular ROI generation was assessed by a previously described assay (Sarafian *et al*, 1994) that primarily measures hydrogen peroxide and hydroxyl radical. Briefly, targets in 48 well plates were washed × 2 with 0.5 ml buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 5 mM $NaCO_3$, 6 mM glucose, 1.2 mM $MgSO_4$ and 1.0 mM $CaCl_2$) and loaded with 40 μM 2,7 dichlorofluorescin diacetate (DCF) in 0.5 ml buffer. After incubating in the dark for 20 min at room temperature, cells were washed × 2, sealed and kept in the dark until reading. DCF fluorescence was measured over a 3 h time period at 30 min intervals with a cytofluor 2300 plate reader set at EA=485 and EM=530. The assay was run with three wells/group and mean fluorescence was calculated. Values were normalized to cell numbers based on maximum propidium iodide fluorescence values in the presence of 160 μM digitonin.

Assay for intracellular glutathione (GSH) levels

As previously described (Sarafian *et al*, 1994), GSH levels were assayed with the fluorescent reagent monochlorobimane (MCB) and cytofluor 2300 set at Ex=395, Em=460. Following DCF measurements, buffer was removed and 250 μl of fresh buffer containing 40 μM MCB was added. Fluorescence was measured after 15 min and values normalized to cell numbers as above.

Thymine glycol determination

As previously described (Zimmerman *et al*, 1989), cell pellets were lysed in 10 mM Tris-HCl:1 mM EDTA (pH 8.0):0.1% sodium dodecyl sulfate and incubated with RNase A (0.1 mg/ml) for 1 h and proteinase K (0.1 mg/ml) for 1 h. The DNA was purified by extractions with phenol:chloroform:isoamylalcohol (24:24:1), and chloroform:isoamylalcohol (24:1), followed by precipitation in 70% alcohol.

The DNA was dissolved in PBS and concentration and purity determined spectrophotometrically. The ELISA for quantification of thymine glycols in DNA was performed using the monoclonal antibody GB10/C5, a generous gift from Dr S.A. Leadon.

Western blot determination

Transfected cells were washed once with cold PBS. Five million cells were lysed in 50 μl of lysis buffer (1% triton x 100, 0.5% NP40, 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM NaF and 0.2 mM PMSF). Cell lysates were cleared at 14 000 rpm for 15 min at 4°C. Twenty-five μg of protein from each sample was boiled for 5 min in 1 $\times$ SDS gel-loading buffer (125 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 1% beta-mercaptoethanol and 0.006% bromophenol blue). Proteins were separated by 12.5% SDS – PAGE and transferred onto PUDF membrane. The membrane was blocked 1 h at room temperature in 3% BSA, 5% non-fat dried milk, 10 mM Tris, pH 7.5, 100 mM NaCl and 0.1% Tween 20). After four washes in TBS-T buffer, the membrane was incubated with 0.5 μg/ml rabbit anti-human BCL-2 antibody (kind gift from Dr John Reed, La Jolla, CA) for 1 h. After four more washes, the membrane was overlayed with 1 μg/ml HRP-labelled goat anti-rabbit IgG antibody (Amersham) and the BCL-2 protein band was detected with an ECL system.

Statistics

Significant differences between groups was determined by the *t*-test using the Bonserroni correction for multiple comparisons.

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