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Inhibition of receptor-mediated apoptosis upon Bcl-2 overexpression is not associated with increased antioxidant status

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ABSTRACT

Bcl-2 is reported to augment the antioxidant capacity of cells and this is hypothesized to contribute to the anti-apoptotic activity of this oncoprotein. We generated a number of stable Jurkat cell lines expressing varying levels of Bcl-2, and showed a strong correlation between Bcl-2 levels and resistance to Fas-mediated apoptosis. While individual differences could be detected, there was no overall correlation between Bcl-2 and the expression and activity of superoxide dismutases, catalase, glutathione peroxidases, thioredoxin, thioredoxin reductases, and peroxiredoxins. Cells transfected with Bcl-2 averaged 70% more glutathione than parental cells, but there was no correlation between glutathione and resistance to apoptosis. This challenges the hypothesis that the anti-apoptotic properties of Bcl-2 are linked to a global increase in antioxidant status.

Bcl-2 is an oncoprotein that is overexpressed in many hematological malignancies [1]. There is considerable interest in the anti-apoptotic activity of Bcl-2, in particular, its ability to complex and inhibit pro-apoptotic members of the Bcl-2 family [2]. Additional mechanisms have also been explored, with the potential importance of the cellular redox environment in susceptibility to apoptosis leading Hockenbery et al. to propose that Bcl-2 may function as an antioxidant [3]. This theory was supported by the initial observation that Bcl-2 protected cells from apoptosis induced by H_2O_2 and menadione [3]. Complementary findings indicated that Bcl-2 could prevent the accumulation of reactive oxygen species [4]. Furthermore, the authors demonstrated that cells overexpressing Bcl-2 had higher levels of the antioxidant glutathione, thus providing a mechanism by which Bcl-2 could provide antioxidant defense.

Since these original findings, several studies have reported that Bcl-2 overexpression enhances the antioxidant capacity of the cell [5–7], and it is hypothesized that antioxidant properties contribute to the anti-apoptotic action of Bcl-2. In particular, a substantial body of literature has reported a link between Bcl-2 expression and the level of intracellular glutathione [6,8–16], and a concomitant increase in the concentration of protein thiols [8,11]. It is hypothesized that a reducing environment can block the induction of apoptosis by scavenging reactive oxygen species, and there are reports showing that Bcl-2 overexpressing cells can be sensitized to apoptosis by glutathione depletion [9,17]. In addition to glutathione, the overexpression of Bcl-2 has been shown to increase the activity of the major antioxidant enzymes superoxide dismutase [8,12,13,18,19], catalase [13,18–21], and glutathione peroxidase [12,19]. The overexpression of these antioxidant enzymes has been reported to protect cells from apoptosis [22–24].

Various studies have also challenged the hypothesis that Bcl-2 has an antioxidant function. It has been demonstrated that Bcl-2 can protect cells from apoptosis in hypoxic conditions, potentially precluding the involvement of reactive oxidants [25,26]. Related studies have shown that the protection Bcl-2 provides from apoptotic stimuli is independent of any alterations in cellular redox [27,28]. We have also shown that while Bcl-2 blocks apoptosis associated with oxidative stress it does not actually protect cellular constituents from oxidation [29]. Furthermore, the effect of Bcl-2 on both antioxidant enzymes and glutathione metabolism may be cell-line dependent [8,13,30], arguing against a universal model in which Bcl-2 overexpression enhances the antioxidant capacity to promote cell survival.

Abbreviations: BSO, L-buthionine *S,R*-sulfoximine; Cat, catalase; DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; DPI, diphenyleneiodonium; DTNB, 5,5′-dithiobis 2-nitrobenoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; Gpx, glutathione peroxidase; Prx, peroxiredoxin; SOD, superoxide dismutase; Trx, thioredoxin; TrxR, thioredoxin reductase; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.

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In the current work we generated a range of Jurkat T-lymphoma cell lines expressing different levels of Bcl-2 in order to determine whether Bcl-2 expression correlates with antioxidant status. Our studies revealed that Bcl-2 consistently increased glutathione levels, however, this change did not correlate with protection from Fas-mediated apoptosis.

Materials and methods

Materials. Culture media and transfection reagents were obtained from GIBCO BRL, Invitrogen, New Zealand. Protease inhibitor cocktail was from Roche Diagnostics, Germany. Mouse anti-Bcl-2 antibody (clone Bcl-2-100) was purchased from Zymed Laboratories Inc., mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (clone 6C5) from Research Diagnostics Inc., and sheep anti-mouse Ig-HRP from Amersham Biosciences, UK. All other antibodies were from AbFrontier, Korea. Diphenyleneiodonium (DPI) and L-buthionine *S,R*-sulfoximine (BSO) were from Sigma–Aldrich, USA. Human activating Fas antibody (clone CH-11) was from Upstate Biotechnology, USA. Caspase substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) was purchased from Peptide Institute Inc., Japan.

Cell culture. Human Jurkat T-lymphoma cells were acquired from ATCC (Rockville, MD, USA) and cultured at 37 °C and 5% CO_2/air in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in antibiotic free media at 5×10^6/ml for 1 h prior to treatment. Anti-Fas antibody (50 ng/ml) was added to the cells and samples taken hourly for determination of apoptosis. In some experiments cells were incubated at a concentration of 1×10^6/ml with 200 μM BSO for 18 h, or with 10 μM DPI for 1 h, before being concentrated to 5×10^6/ml.

pCIneo-bcl2 and pCIneo-GFP construction and stable transfection of Jurkat cells. Full-length human Bcl-2 cDNA (from Professor Suzanne Cory, Walter and Eliza Hall Institute) was subcloned into the mammalian expression vector pCIneo (Promega, USA). Jurkat cells were transfected with Lipofectamine 2000 according to manufacturer's instructions. Cells were selected in RPMI + FBS containing G418 at 700 μg/ml. Stable clones were selected by serial dilution over 5–10 passages and maintained in RPMI 1640 media supplemented with 10% FBS and 350 μg/ml G418. GFP expression in transfected Jurkat cells was determined with a FACSCalibre flow cytometer (Becton Dickinson, Mountain View, CA).

Protein assay and Western blotting. Cells were harvested and resuspended in extract buffer (40 mM HEPES, 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% CHAPS, pH 7.4) containing protease inhibitors. Protein concentrations were determined using the detergent compatible Bio-Rad DC protein assay. Twenty micrograms of total protein was separated by SDS–PAGE and blotted onto Hybond-P PVDF transfer membrane. Immunoreactive bands were visualized by exposure to Kodak X-OMAT K X-ray film following chemiluminescence detection of a horseradish–peroxidase-coupled secondary antibody. X-ray films were scanned using a Fluor-S scanner and bands quantified using Quantity One software (Bio-Rad Laboratories, CA). For each blot Jurkat levels were set to a value of 1 and clones were expressed as fold increase of Jurkat protein intensity.

Determination of antioxidant enzyme activity. Superoxide dismutase activity was measured by employing a superoxide (O_2^-) generating system (xanthine/xanthine oxidase) to reduce a water-soluble tetrazolium dye (WST-1) in the presence of a range of concentrations of superoxide dismutase [31]. Mn-SOD activity was determined in the presence of 3 mM KCN. Catalase activity was determined by measuring the decomposition of H_2O_2 at 240 nm [32]. A coupled spectrophotometer assay involving glutathione reductase was utilized to monitor glutathione peroxidase activity [33]. Thioredoxin reductase activity was determined by measuring the NADPH-dependent reduction of the thiol-reactive probe 5,5′-dithiobis (2-nitrobenoic acid) (DTNB) [34]. Intracellular glutathione levels were measured by HPLC following derivatization with dansyl chloride. Cells were harvested and washed with PBS before resuspension in lysis buffer containing 2.5 mM iodoacetate. The pH was corrected to 8.5 with lithium hydroxide and extracts incubated for 30 min. Dansyl chloride was added and the extracts incubated for a further 60 min in the dark. Following extraction with chloroform, HPLC analysis was performed as described previously [35].

Determination of caspase activity. Caspase activity was measured by resuspending cell pellets in 100 μl of a pH 7.25 buffer containing 100 mM HEPES, 10% sucrose, 5 mM dithiothreitol, 0.1% NP-40, 0.1% CHAPS, and 50 μM DEVD-AMC. The rate of release of fluorescent AMC was monitored at Ex/Em of 390/460 nm.

Statistics. Values are shown as means and standard errors of three or more independent experiments, and all blots are representative of at least three independent experiments. Statistical analyses were performed with the software package SigmaStat (Systat, San Rafael, CA, USA).

Results

Generation of Bcl-2 transfected Jurkat T-lymphoma cell lines

Sixteen different Bcl-2 stable transfectants were selected and protein levels were measured by Western blotting. Expression ranged from 2- (B40) to 50- (B9) fold above that of parental Jurkat cells (Fig. 1). Six pCIneo vector stable transfectants were selected as controls. Transfectants were checked at various times throughout the study to ensure that Bcl-2 protein levels did not significantly change. The anti-apoptotic functionality of Bcl-2 was tested by measuring caspase activation following stimulation of the Fas pathway. The transfected cells were stimulated with 50 ng/ml of anti-Fas antibody and caspase-3-like DEVDase activity was measured every 60 min during the first 4 h and the rate of caspase activation calculated. For each transfectant the linear rate of activation

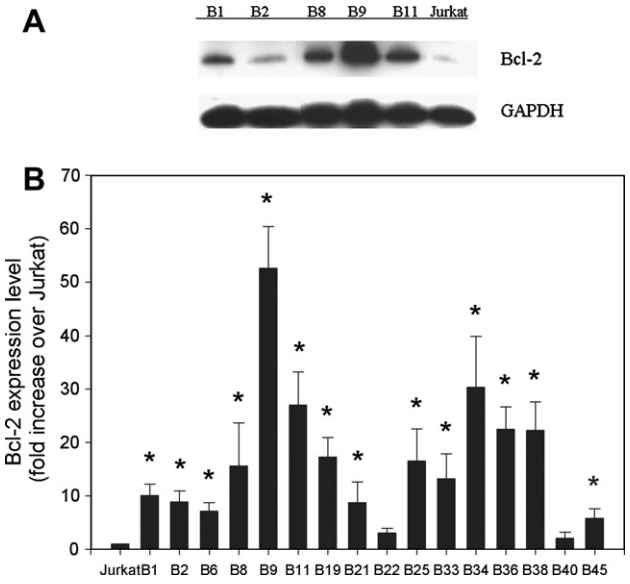

Fig. 1. Bcl-2 expression in a series of transfected Jurkat clones. (A) Western blot of Bcl-2 and GAPDH expression in whole cell lysates of individual clones. (B) Bcl-2 expression in clones relative to parental Jurkat cells. Results represent means ± SE from at least three independent blots obtained from cells harvested at different periods during the time of the study. $^*p < 0.05$ versus Jurkat control.

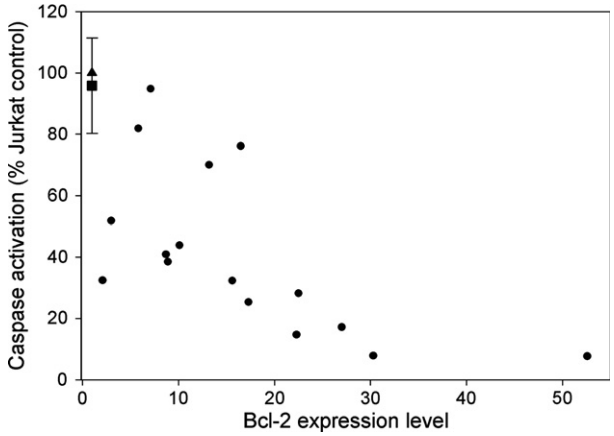

Fig. 2. Correlation of Bcl-2 expression with caspase activation during Fas-mediated apoptosis. Bcl-2 expression and caspase activity following addition of anti-Fas antibody was assessed in three independent experiments for (■) Jurkat cells, (●) pCIneo-bcl2 clones, (▲) pCIneo vector clones. The correlation coefficient for Bcl-2 level versus caspase activation was −0.63 for the 16 pCIneo-bcl2 clones (Pearson Product Moment Correlation, $P = 0.01$). All cell lines were assayed in duplicate, results are the mean of at least three separate experiments. For the Jurkat cells error bars are shown to represent the standard error.

was calculated as a percentage of that obtained for parental Jurkat cells. Caspase activation rates varied greatly between Bcl-2 clones, ranging from 8% up to 96% (Fig. 2). There was a direct correlation between Bcl-2 levels and resistance to apoptosis. The vector-only control clones showed no resistance to Fas-induced apoptosis, averaging 96% of the parental cells.

Bcl-2 expression does not correlate with increased antioxidant enzyme expression or activity

Lysates of Bcl-2 transfected clones were prepared and antioxidant enzyme expression (Table 1) and activity (Table 2) was measured. Antioxidant enzyme expression varied widely between the clones. For example, the B2 clone showed increased expression of peroxiredoxin 2 and 6 compared to parental cells, while B36 and B38 clones showed low levels of thioredoxin reductase 1 and 2 expression. The antioxidant enzyme activity among the clones was not as variable. However, the B2 clones stood out as having 120% superoxide dismutase and 70% glutathione peroxidase activity of parental Jurkat cells. Despite these small fluctuations in individual clones there was no correlation between Bcl-2 expression and antioxidant enzyme expression or activity.

Low-level Bcl-2 overexpression increases intracellular glutathione

We measured intracellular glutathione levels in all of the selected clones. The level of glutathione in the Bcl-2 transfectants was 170% that of parental cells (Fig. 3A). Cells transfected with vector alone also showed increased glutathione, but there was a significant difference from those expressing Bcl-2. Although glutathione levels increased in the Bcl-2 transfectants, there was no direct correlation between Bcl-2 expression and glutathione levels (Fig. 3B). Indeed, some of the clones with the highest glutathione had relatively low levels of Bcl-2. As an additional control we generated six Jurkat cell lines expressing varying levels of green fluorescent protein (GFP). GFP transfectants were selected in the same manner as the Bcl-2 transfectants, and glutathione analysis

Table 1
Bcl-2 expression does not alter expression of antioxidant enzymes

	Cell lines						
	N1	N2	B1	B2	B9	B36	B38
Bcl-2	1.1	1.3	10.1	8.9	52.6	22.5	22.3
Gpx1	1.33 ± 0.18	1.31 ± 0.14	1.28 ± 0.30	0.92 ± 0.27	0.79 ± 0.25	1.29 ± 0.19	1.25 ± 0.24
Gpx3	0.67 ± 0.15	0.81 ± 0.08	1.08 ± 0.05	1.11 ± 0.07	0.97 ± 0.27	0.79 ± 0.13	0.97 ± 0.11
Gpx4	1.07 ± 0.20	1.02 ± 0.19	1.21 ± 0.23	1.25 ± 0.13	1.13 ± 0.24	1.27 ± 0.30	1.19 ± 0.34
Prx1	1.06 ± 0.42	0.98 ± 0.25	0.95 ± 0.33	0.89 ± 0.64	0.95 ± 0.50	0.87 ± 0.47	0.6 ± 0.21
Prx2	1.41 ± 0.17	1.83 ± 0.30	1.28 ± 0.32	1.59 ± 0.51	1.4 ± 0.29	1.12 ± 0.36	1.17 ± 0.06
Prx3	1.09 ± 0.19	0.99 ± 0.28	0.9 ± 0.21	0.81 ± 0.13	1.04 ± 0.16	1.3 ± 0.38	1.16 ± 0.47
Prx4	0.95 ± 0.07	0.54 ± 0.22	0.67 ± 0.19	0.58 ± 0.08	0.85 ± 0.13	0.89 ± 0.17	0.9 ± 0.32
Prx5	1.14 ± 0.41	1.07 ± 0.59	0.54 ± 0.05	0.52 ± 0.03	1.01 ± 0.20	0.9 ± 0.02	0.56 ± 0.03
Prx6	1.26 ± 0.34	1.27 ± 0.47	1.82 ± 0.4	1.94 ± 0.48	1.84 ± 0.45	1.32 ± 0.41	0.89 ± 0.33
Trx1	1.07 ± 0.27	0.98 ± 0.29	1.10 ± 0.33	0.89 ± 0.07	1.02 ± 0.18	1.03 ± 0.30	1.25 ± 0.36
TrxRed1	1.13 ± 0.09	1.09 ± 0.22	1.06 ± 0.23	0.83 ± 0.20	0.92 ± 0.27	0.73 ± 0.32	0.83 ± 0.14
TrxRed2	0.98 ± 0.31	1.08 ±0.40	1.03 ± 0.29	1.17 ± 0.08	0.89 ± 0.19	0.99 ± 0.16	0.82 ± 0.15

Antioxidant expression in pCIneo clones (N1 and N2) and Bcl-2 clones (B1, B2, B9, B36, and B38) is stated relative to parental Jurkat cells which are set to 1. Values represent means ± SE of at least three independent experiments. GPx, glutathione peroxidase; Prx, peroxiredoxin; Trx, thioredoxin; TrxRed, thioredoxin reductase. There was no significant correlation ($p > 0.05$) between Bcl-2 and any of the listed antioxidants as performed by Pearson's paired comparison of variables.

Table 2
Bcl-2 expression does not enhance antioxidant activity

Antioxidant enzyme activity	Cell lines							
	Jurkat	N1	N2	B1	B2	B9	B36	B38
Cu, Zn-SOD (U/mg)	350 ± 27	388 ± 20	357 ± 35	377 ± 31	424 ± 20	390 ± 21	380 ± 18	356 ± 21
Mn-SOD (U/mg)	110 ± 29	137 ± 19	113 ± 12	140 ± 29	137 ± 19	124 ± 17	123 ± 22	90 ± 39
Gpx (mU/mg)	132 ± 5	113 ± 18	147 ± 13	114 ± 21	93 ± 17	111 ± 15	101 ± 16	116 ± 9
Cat (U/mg)	8.70 ± 1.3	9.21 ± 0.49	7.91 ± 1.3	8.74 ± 1.3	8.02 ± 1.7	9.13 ± 1.8	7.91 ± 0.81	7.19 ± 0.61
TrxRed (ΔmAU_{412nm}/min)	81 ± 13	86 ± 9	87 ± 13	95 ± 16	78 ± 7	84 ± 2	79 ± 13	72 ± 5

Values represent means ± SE of at least three independent experiments. SOD, superoxide dismutase; GPx, glutathione peroxidase; Cat, catalase; TrxRed, thioredoxin reductase. There was no significant correlation ($p > 0.05$) between Bcl-2 and any of the antioxidant enzyme activities as performed by Pearson's paired comparison of variables.

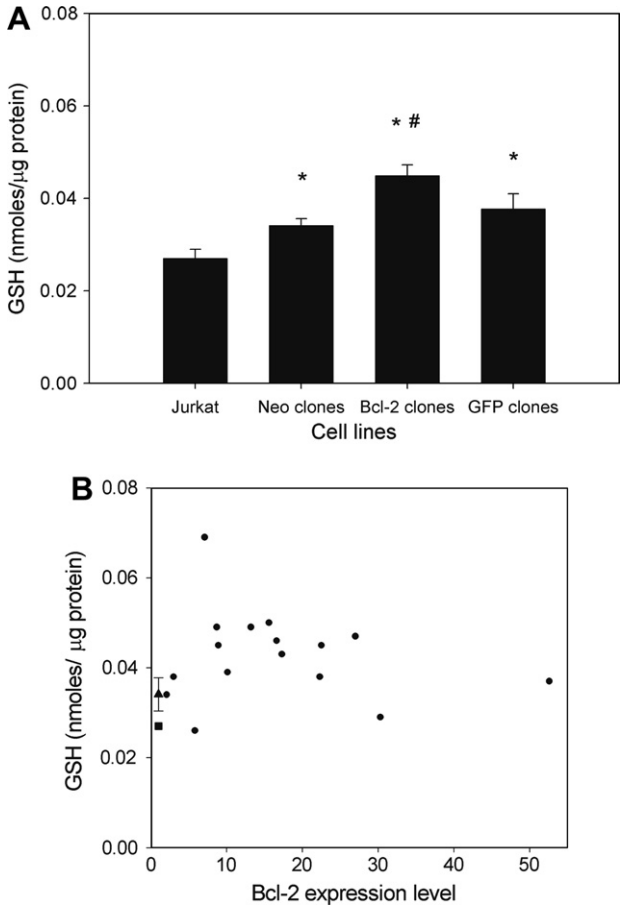

Fig. 3. Analysis of glutathione levels in Bcl-2 clones. (A) Glutathione levels in parental Jurkat cells, six pCIneo vector control, 16 pCIneo-bcl2, and six pCIneo-GFP clones. Each clone was tested in duplicate and the results are means ± SE of 6, 16, and 6 clones respectively. Glutathione levels were measured in the parental Jurkat cells in six different experiments. $^{*}p < 0.05$ versus Jurkat control; $^{\#}p < 0.05$ versus Neo control. (B) Correlation of Bcl-2 versus glutathione levels for the 16 pCIneo-bcl2 clones. There was no significant correlation between Bcl-2 and glutathione levels for the 16 pCIneo-bcl2 clones (Pearson Product Moment Correlation, $r = 0.16$, $P = 0.55$).

undertaken. GFP transfectants showed a 40% increase in glutathione (Fig. 3A).

Increased glutathione does not protect transfectants against Fas-induced apoptosis

There was no correlation between intracellular glutathione and caspase activation following Fas stimulation in the Bcl-2 transfectants (Fig. 4A). Intracellular glutathione levels were also lowered and the sensitivity of cells to Fas-mediated apoptosis was measured. Glutathione depletion was achieved by either incubating cells for 18 h in the presence of the γ-glutamylcysteine synthase inhibitor buthionine sulfoximine (BSO), or by exposing cells to diphenyleneiodonium (DPI), which causes increased glutathione efflux [36]. DPI was used to lower glutathione to levels similar to the parental Jurkats, while BSO exposure caused a more dramatic reduction of up to 90% of the original level of glutathione (Fig. 4B). In each system, the reduction of intracellular glutathione had no significant effect on sensitivity to Fas-mediated apoptosis (Fig. 4C).

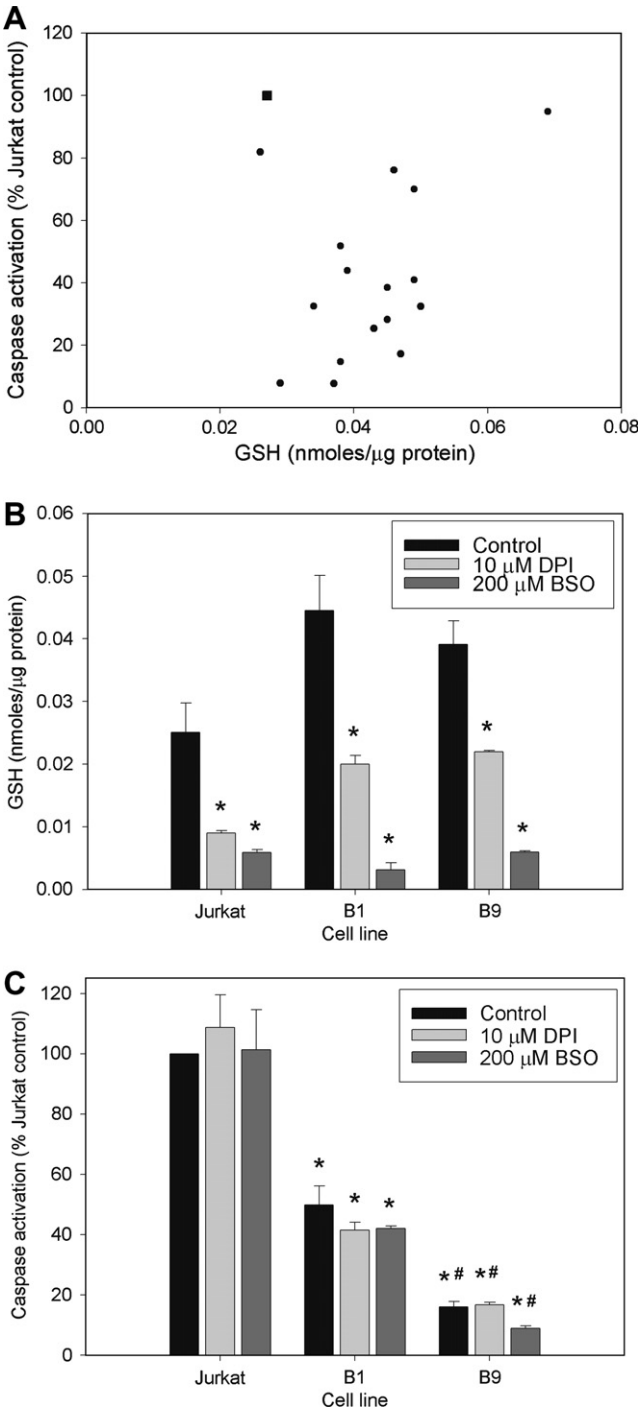

Fig. 4. Intracellular glutathione does not correlate with sensitivity to Fas-mediated apoptosis. (A) Intracellular glutathione concentration versus caspase-3 activation. (■) Jurkat control, (●) pCIneo-bcl2 clones. There was no significant correlation between glutathione levels and caspase activation for the 16 pCIneo-bcl2 clones (Pearson Product Moment Correlation, $r = 0.40$, $P = 0.13$). (B) Intracellular glutathione was measured after 18 h incubation in the presence of 200 μM BSO, or after 1 h incubation with 10 μM DPI. $^{*}p < 0.05$ versus untreated control. (C) Treated cells were incubated with anti-Fas at 50 ng/ml and caspase activation rates were measured as described in Materials and methods. Results are presented as means ± SE of three independent experiments, expressed as a percentage of the caspase activation observed in untreated Jurkat cells. $^{*}p < 0.05$ versus Jurkat control; $^{\#}p < 0.05$ versus B1 clone.

Discussion

The objective of this study was to determine if Bcl-2 expression augmented antioxidant capacity in cultured cells, and if this could be related to the anti-apoptotic properties of Bcl-2. Using a large number of stable transfectants we clearly demonstrated a direct correlation between the level of Bcl-2 expression and protection against Fas-mediated apoptosis. Variations in antioxidant

expression were detectable in individual clones, but there was no relationship between Bcl-2 and antioxidant levels, and hence no relationship between antioxidant levels and inhibition of apoptosis. The most consistent phenomenon was increased glutathione in Bcl-2 transfected cells, confirming several earlier studies [8–10,37]. However, there was a large variability in glutathione levels between the Bcl-2 clones and the increases did not correlate with transgene expression, highlighting the importance of using multiple clones. Glutathione levels were also increased (although to a lesser extent) in cells transfected with the vector alone or in GFP transfectants. This observation warrants further investigation as it suggests that the transfection process itself may lead to selection of clones with higher glutathione levels.

Glutathione efflux is proposed to be an important step in the progression of apoptosis in response to both oxidative and non-oxidative stimuli [38,39]. It is less clear whether increased concentrations of glutathione can make cells resistant to apoptosis. Various studies have attempted to understand the role glutathione plays in Bcl-2-dependent resistance by depleting glutathione and then assessing apoptosis sensitivity [37,40]. A potential complication exists when glutathione-depleted cells are challenged with oxidative stimuli because overwhelming stress and necrotic cell death may occur. Under such conditions it is not surprising that Bcl-2 provides little protection. The best means of determining if increased glutathione in response to Bcl-2 overexpression provides protection is to lower levels to that seen in untransfected cells. An alternate approach has been to elevate glutathione levels with large extracellular concentrations of reduced glutathione or *N*-acetylcysteine [41]. It will be important to determine that the effect of these compounds to inhibit apoptosis is specific to their ability to increase intracellular glutathione rather than an effect at the cell surface on the initiation of receptor-mediated signaling pathways.

In addition to glutathione, previous studies have failed to reach a consensus as to whether or not the overexpression of Bcl-2 enhances the activity of antioxidant enzymes. The disparity may reflect differences in the cell lines used [8,13,30], with investigators who have used human lymphoma cell lines typically coming to the conclusion that there are no antioxidant aspects to Bcl-2 function [42]. However, it also has to be considered that many of the studies reporting Bcl-2 antioxidant properties have been performed using only a single Bcl-2 clone, and these need to be interpreted with caution. The random variations seen in this study highlight the importance of using multiple clones. The selection procedure used to isolate Bcl-2 clones, which involves exposing cells to high levels of geneticin, may promote the survival of clones with elevated antioxidant status, independent of the transgene involved. Finally, there is also the problem of insertional variation whereby the phenotype of the clone maybe altered depending on the site at which the transgene has been inserted.

In summary, our results do not exclude the possibility that Bcl-2 has a specific and localized effect on redox signaling pathways that influence the progression of apoptosis. However, consistent with our previous study showing a similar degree of oxidative damage in Bcl-2 transfectants and parental Jurkat cells exposed to hydrogen peroxide [29], it is clear that anti-apoptotic properties of Bcl-2 in Jurkat lymphoma cells do not involve a global increase in antioxidant activity.

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