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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

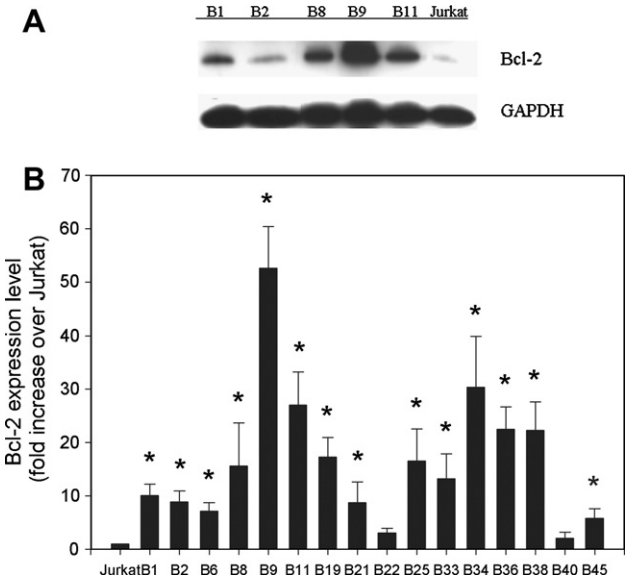

Fig. 1. Bcl-2 expression in a series of transfected Jurkat clones. (A) Western blot of Bcl-2 and GAPDH expression in whole cell lysates of individual clones. (B) Bcl-2 expression in clones relative to parental Jurkat cells. Results represent means ± SE from at least three independent blots obtained from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