RESEARCH COMMUNICATION

Bcl-x_L overexpression attenuates glutathione depletion in FL5.12 cells following interleukin-3 withdrawal

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Bcl-x_L and *bax* are *bcl-2*-related genes whose protein products either inhibit or promote apoptosis. Oxidative damage, including the loss of glutathione, has been implicated in the induction of apoptosis. The ability of the Bcl proteins to affect GSH was assessed in control, *bax*- and *bcl-x_L*-transfected FL5.12 cells [an interleukin (IL)-3-dependent murine prolymphocytic cell line]. Overall levels of GSH were approximately the same in control and *bcl-x_L* transfectants during the 6 h incubation period, although levels increased in *bcl-x_L* transfectants 24 h after replating. GSH in cells overexpressing *bax* was reduced by $\sim 36\%$. There were no consistent differences between these cell lines in the activities of superoxide dismutase, catalase, glutathione peroxidase or glutathione reductase. Following IL-3 withdrawal, a condition known to cause apoptosis in these cells, a rapid loss of intracellular GSH occurred in control and *bax* transfectants, which preceded the onset of apoptosis. GSH depletion could not be attributed to intracellular oxidation but rather seemed to occur due to a translocation out of the cell. Cells overexpressing *bcl-x_L* did not lose significant amounts of GSH upon withdrawal of IL-3, and no apoptosis was evident. These results suggest a possible role for GSH in the mechanism by which *bcl-x_L* prevents cell death.

INTRODUCTION

Apoptosis is a form of cell death that differs from necrosis by distinct morphological and biochemical features, including chromatin condensation, plasma blebbing, oligonucleosomal DNA fragmentation and, finally, the breakdown of the cell into smaller units (apoptotic bodies) that in most tissues are phagocytosed by nearby cells [1–3]. Apoptosis can be initiated by a variety of stimuli, including growth-factor or hormone withdrawal, glucocorticoids, oxidants and ionizing radiation [1]. In addition, this process eliminates unwanted cells during development as well as removing mutated and potentially cancerous cells.

The *bcl-2* gene product, originally discovered because of its involvement in the t(14:18) translocation present in many human B-cell lymphomas that leads to transcriptional deregulation and overproduction of this protein, is a hydrophobic 26 kDa protein that resides in cellular membranes of the nuclear envelope, endoplasmic reticulum and mitochondrial outer membrane [4]. *bcl-2*, the first negative regulator of apoptotic cell death to be identified [5,6], has been widely studied and shown to protect cells from apoptosis induced by a broad range of stimuli. *bcl-x_L* is a related gene whose 29 kDa protein product has an almost identical subcellular localization and effects as *bcl-2* [7–9], but has been studied in much less detail.

Substantial evidence suggests that the growing Bcl family of proteins play a critical role in regulating the balance between cell proliferation, differentiation and apoptosis. It has been proposed that the amount of Bcl-2 alone is not critical, but rather that the balance between this protein and Bax, the gene for which encodes a dominant inhibitor of *bcl-2*, is an important regulatory mechanism [10]. Bcl-x_L, however, does not appear to be regulated by heterodimerization with Bax [11], calling into question the prevalence of this putative regulatory mechanism.

Because *bcl-2* overexpression blocks apoptosis and necrosis induced by oxidants, it was hypothesized that it may prevent cell death by decreasing the oxidant status of the cell [12–15]. Although Bcl-2 and Bcl-x_L do not display any direct antioxidant capacity, various antioxidant enzyme systems, such as glutathione peroxidase and superoxide dismutase (SOD), can replace Bcl-2 and prevent apoptosis. In contrast, several recent reports showed that *bcl-2* overexpression can inhibit oxygen-independent apoptosis [16,17]. These observations suggest that oxidants are unlikely to be essential mediators of this type of cell death and that *bcl-2* inhibits apoptosis by other mechanism that may not require oxygen [18]. Collectively, the association among oxidants, the Bcl-2 proteins and apoptosis remains largely unknown.

There has been an interesting correlation in some, but not all, cells with the levels of glutathione and the cells' ability to resist or undergo apoptosis. One explanation for the apparent discrepancies in the role of GSH and oxidants is the report that the oxidative tonus of cells exposed to anti-Fas antibody to induce apoptosis is increased by the stimulation of GSH efflux and not by the formation of reactive oxygen species (ROS) [19]. Supplementing these cells with GSH did not prevent apoptosis, possibly due to rapid efflux. On the other hand, antioxidants may provide some protection by slowing oxidative processes that proceed in the absence of adequate GSH [20]. The key factor in apoptosis may, therefore, be GSH, and modification of this cellular reducing agent by either oxidation of extrusion may be critical.

To address this issue further, the present study was designed to measure GSH as well as several antioxidant enzyme systems in FL5.12 cells [an interleukin (IL)-3 dependent murine prolymphocytic cell line] overexpressing either *bcl-x_L* or *bax*. It is shown that all antioxidant enzyme systems were the same in control and transfected cell lines. Following IL-3 withdrawal, a loss of intracellular GSH occurred in control and *bax*-transfected FL5.12 cells that preceded apoptosis. The depletion in GSH was not the result of intracellular oxidation but rather seemed to occur due to an efflux of GSH. Neither apoptosis nor a loss of GSH occurred in the *bcl-x_L* transfectants, which also tended to have higher GSH levels.

Abbreviations used: IL-3, interleukin-3; ROS, reactive oxygen species; SOD, superoxide dismutase; LDH, lactate dehydrogenase.

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MATERIALS AND METHODS

Cell culture

IL-3-dependent murine prolymphoid progenitor cell lines (FL5.12) stably transfected with either a SFFV-NEO control construct, an SFFV-FLAG-bcl-x_L construct or an SFFV-HA-bax construct were used in these studies and have been described previously [11]. FL5.12 transfectants were maintained in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/μl) and 10% (v/v) WEHI-3B-conditioned medium in a CO_2/air (1:19) atmosphere at 37 °C. WEHI-3B cells were grown under similar conditions to produce IL-3-conditioned medium as described previously [21]. Cultures were passaged on alternate days with fresh medium, and cell counts were determined with a T-890 Coulter counter (Coulter, Miami, FL, U.S.A.).

Western-blot assay

Cells were lysed with 1 ml of RIPA buffer [10 mM sodium phosphate, 150 mM NaCl (pH 7.4), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 100 μg/ml PMSF, 30 μl/ml aprotinin and 1 mM sodium orthovanadate] by repeatedly pipetting the cell suspension. The lysed cells were centrifuged at 400 ***g*** for 10 min, and the supernatants were run on reducing SDS/15%-polyacrylamide gels [buffer composition: 4% (w/v) SDS, 20% (w/v) glycerol, 4% (w/v) β-mercaptoethanol, 0.2 M Tris/HCl (pH 6.8) and 0.02% (w/v) Bromophenol Blue]. Protein was transferred on to PVDF membranes and blocked for 1 h. The membrane was then incubated with either polyclonal anti-Bcl-x_L antibodies (1:150 dilution) or polyclonal anti-Bax antibodies (1:2000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 1 h. After membrane washing, horseradish peroxidase-conjugated secondary antibodies were used (1:2000 dilution; Sigma, St. Louis, MO, U.S.A.). Bound antibodies were detected using enhanced chemiluminescence with a kit from Amersham (Arlington Heights, IL, U.S.A.). Protein content was determined by the method of Lowry et al. [22], using BSA as a standard.

Measurement of apoptosis and cell viability

Control, *bcl-x*$_L$- and *bax*-transfected cells (5×10^6 cells/ml) were plated in complete media in the presence or absence of IL-3. After 0.5, 1, 2, 4 and 6 h the cells were pelleted at 300 ***g*** for 10 min at 4 °C. The cells were resuspended in 40 μl of fresh media. Apoptosis was assessed by fluorescence microscopy by mixing 2 μl of Acridine Orange (100 μg/ml), 2 μl of ethidium bromide (100 μg/ml) and 20 μl of the cell suspension. A minimum of 200 cells were counted in at least five random fields. 'Live' apoptotic cells were differentiated from 'dead' apoptotic, necrotic and normal cells by examining the changes in cellular morphology based on distinctive nuclear and cytoplasmic fluorescence [23]. Overall cell viability was also assessed by Trypan Blue exclusion.

Lactate dehydrogenase (LDH) assay

Control, *bcl-x*$_L$- and *bax*-transfected cells (2.5×10^6 cells/ml) were plated in complete media in the presence or absence of IL-3. After 0 and 6 h LDH was analysed using a modified method of Mitchell et al. [24].

Enzymatic assays

Control and *bcl-x*$_L$ transfected cells were plated at a density of 1.25×10^6 cells/ml in 25 cm^2 flasks in media with or without IL-3. After 6 and 24 h, cells were pelleted (400 ***g***, 10 min), and the pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 0.5% (w/v) Triton X-100 and then sonicated for 5 min. Sonicates were spun for 10 min at 15000 ***g***, and the supernatants were either immediately used or stored at −80 °C. All enzymic assays were performed following storage at −80 °C except for glutathione peroxidase, which was conducted immediately. Catalase activity was measured by monitoring the disappearance of H_2O_2 at 240 nm following the method described by Luck [25]. SOD activity was determined by measuring the SOD-mediated increase in the rate of auto-oxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[*c*]-fluorene, using a kit from OXIS International (Portland, OR, U.S.A.). Glutathione reductase activity was assayed spectrophotometrically following NADPH depletion at 340 nm [26]. Glutathione peroxidase activity was determined using a coupled assay in which the rate of H_2O_2-dependent NADPH oxidation at 340 nm was monitored [27]. Total protein in the cellular lysate was determined as before.

Glutathione determination

Total glutathione (GSH + GSSG) and GSSG levels were determined using a modified method of Neuschwander-Tetri and Roll [28]. Control, *bax*- and *bcl-x*$_L$-transfected cells (5×10^6 cells/ml) were collected following incubation in media with or without IL-3 and were centrifuged for 10 min at 300 ***g*** at 4 °C. The pellet was resuspended in 1 ml of EDTA (2 mM) and was sonicated for 2 min. Total glutathione was determined in both the cell lysate (intracellular) and supernatant (culture media) by adding 83 μl of 25 mM NaH_2PO_4 (pH 7.0 to 250 μl of lysate and supernatant. An additional 250 μl of cell lysate was mixed with 83 μl of *N*-ethylmaleimide for intracellular GSSG measurements. All samples (200 μl each) were then mixed with 200 μl of 25 mM dithiothreitol (in 25 mM NaH_2PO_4, pH 7.0) followed by 100 μl Tris buffer (pH 8.5) and were incubated on ice for 30 min. Following addition of 0.5 ml of 2.5% (w/v) sulphosalicyclic acid, the samples were centrifuged for 10 min at 600 ***g*** at 4 °C. An aliquot (200 μl) of supernatant was mixed with 200 μl of *o*-phthalaldehyde (5 mg/ml in 0.4 M potassium borate solution, pH 9.9) and incubated for 2 min at 25 °C. The mixture was neutralized, and samples either were kept on ice in the dark and used immediately or were stored at −80 °C. Derivatized samples were analysed as described previously [29].

Statistics

Data are expressed as means ± S.E.M. Comparisons between groups were done with a one-way analysis of variance followed by Student–Newman–Keul's test. A P value of less than 0.05 was considered to be significant.

RESULTS

Overexpression of Bcl-x_L, a 29–30 kDa protein, and Bax, a 24 kDa protein, in FL5.12 cells was shown previously [11] and confirmed here by Western-blot analysis (results not shown). Control Fl5.12 cells expressed a significant amount of endogenous Bax, but very low levels of endogenous Bcl-x_L.

Control and *bax* transfectants subjected to IL-3 deprivation for 6 h began to display a slight but significant reduction in the percentage of viable cells (as assessed by Trypan Blue), from 98% to ~83 and 87% respectively (results not shown). In contrast, *bcl-x*$_L$ transfectants subjected to IL-3 deprivation demonstrated resistance to cell death, with essentially no loss in viability (98% viable cells) over the 6 h culture period studied. The percentage of total LDH found in the media at time 0 from control, *bcl-x*$_L$ and *bax* cells was 8, 13 and 7% respectively. This

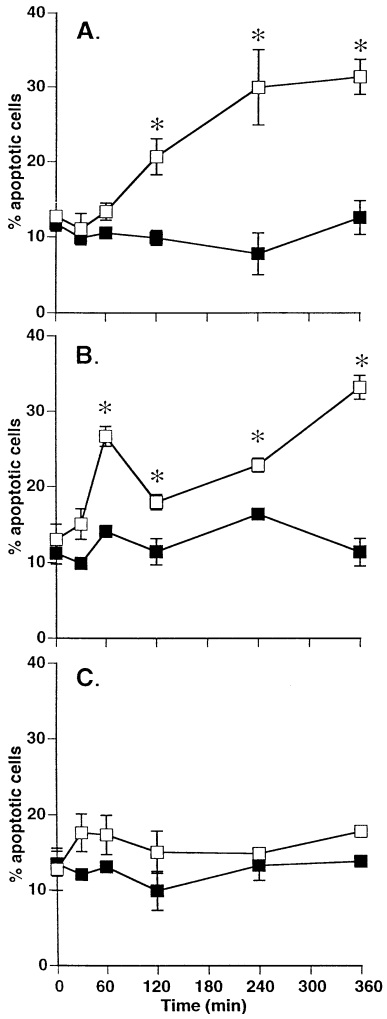

Figure 1 Apoptosis in FL5.12 cells following IL-3 withdrawal

(**A**) Control; (**B**) *bax*- and (**C**) *bcl-x_L*-transfected cells (5×10^6 cells/ml) were plated in medium containing IL-3 (■) or in medium lacking IL-3 (□). After 0.5, 1, 2, 4 and 6 h, apoptosis was determined as described in the Materials and methods section. A minimum of 200 cells were counted per slide in at least five random fields, and the percentage of 'live' apoptotic cells per total number of cells was determined. Results are means ± S.E.M. ($n = 3$–6). *Significantly different from IL-3-supplemented counterparts ($P < 0.05$).

was unchanged 6 h after withdrawal of IL-3 (12, 11 and 6 % respectively).

Control and *bax* transfectants showed a moderate but significant increase in the percentage of apoptotic cells beginning 1–2 h following removal of IL-3 (Figures 1a and 1B). The number of apoptotic cells increased with time, and at 6 h the percentages of apoptotic cells were about 3-fold higher than cells with IL-3, for control and *bax*-transfected cells. In contrast with these results, the withdrawal of IL-3 did not increase the extent of apoptosis in cells that overexpressed *bcl-x_L* (Figure 1c).

In order to determine whether *bcl-x_L* overexpression changed the basal activities of antioxidant enzymes in FL5.12 cells, catalase, SOD, glutathione reductase and glutathione peroxidase activities were measured 6 and 24 h after cells were plated (Table 1). Catalase activity was increased 1.5-fold in cells overexpressing *bcl-x_L* relative to controls at 6 h after subculturing. However, at 24 h this activity decreased to levels that did not vary significantly from those of controls. There were no differences between *bcl-x_L* and control transfectant cells in all the other antioxidant enzyme systems examined at either 6 or 24 h.

Table 1 Activities of antioxidant enzymes in control and *bcl-x_L*-transfected FL5.12 cells

Cells were plated at a density of 1.25×10^6 cells/ml in 25 cm^2 flasks, and cell lysates were extracted at 6 and 24 h. Data are means ± S.E.M. Values for enzyme activities are given in units/mg of protein extract ($n = 4$–6). Units are defined as follows: glutathione reductase (GR), nmol of NADPH oxidized/min; SOD, SOD-mediated increase in the auto-oxidation of 5,6,6A,11B-tetrahydro-3,9,10-trihydroxybenzo[*c*]fluorene; catalase, μmol of H_2O_2 consumed/min; glutathione peroxidase (GPx), nmol of NADPH oxidized/min. * Significantly different from Control ($P < 0.05$).

Time (h)...	Activity 6		24	
Enzyme	Control	*bcl-x_L*	Control	*bcl-x_L*
GR	9.3 ± 0.8	12.4 ± 1.8	7.8 ± 0.9	10.2 ± 0.8
SOD	15.0 ± 1.9	12.5 ± 3.2	5.0 ± 0.7	6.6 ± 0.7
Catalase	37.7 ± 3.7	57.4 ± 4.7*	33.3 ± 3.4	33.8 ± 2.9
GPx	38.6 ± 6.7	41.0 ± 4.0	36.3 ± 5.0	42.3 ± 7.0

In the presence of IL-3, levels of intracellular GSH throughout the 6 h incubation were the same between control and *bcl-x_L*-transfected cells (Figures 2a and 2c). Combining all of these data points yielded levels of 14.2 ± 1.2 and 13.7 ± 1.0 nmol of total GSH/mg protein for control and *bcl-x_L* cells respectively. Interestingly, GSH levels were significantly increased in *bcl-x_L* cells, compared with control cells, 24 h after cells were replated (28.0 ± 3.0 and 13.5 ± 1.2 nmol of total GSH/mg of protein for *bcl-x_L* and control cells respectively) and returned to basal levels at 48 h, which is also the 'zero' time point. In contrast, in the presence of IL-3, cells overexpressing *bax* displayed significantly lower levels of GSH over the 6 h incubation (9.0 ± 1.9 nmol of total GSH/mg of protein) (Figure 2b).

Following IL-3 withdrawal, total intracellular GSH decreased with time in both control and *bax*-transfected cells (Figures 2a and 2b). A significant reduction of GSH was noted as soon as 1 h after IL-3 was removed from control cells and continued to decline over the 6 h culture period to levels 2.3-fold lower than IL-3-supplemented counterparts (Figure 2a). Similarly, cells overexpressing *bax* showed a significant decrease in GSH content after IL-3 was removed, and at 4 h total GSH levels were decreased by about 50 % (Figure 2b). *bcl-x_L* transfectants did not display any significant loss in GSH content with time, independent of the presence of IL-3 (Figure 2c).

Oxidative stress is one mechanism for GSH depletion [30]. However, no changes in GSSG were detected in control, *bax* or *bcl-x_L* transfectants cultured in the presence or absence of IL-3 (results not shown). Because it is possible that increases in GSSG were not detected because of its rapid reduction by glutathione reductase, this enzyme was inhibited with 1 mM, 1,3-bis-(2-chloroethyl)-1-nitrosurea in control cells deprived of IL-3. After 2 h (a time point where a significant decrease in total intracellular GSH was observed; Figure 2a), glutathione reductase activity was decreased by 87 % [7.4 ± 0.3 (in control cells) compared with 0.9 ± 0.2 (after 2 h) nmol of NADPH oxidized/min per mg of protein]. However, irrespective of glutathione reductase inhibition, GSSG was still not increased, suggesting that the GSH lost is not present in the disulphide form within the cell.

Previous studies conducted in T-lymphocytes showed that apoptosis induced by anti-FAS/APO-1 antibody caused an efflux of GSH into the extracellular media [19]. A rapid loss of about

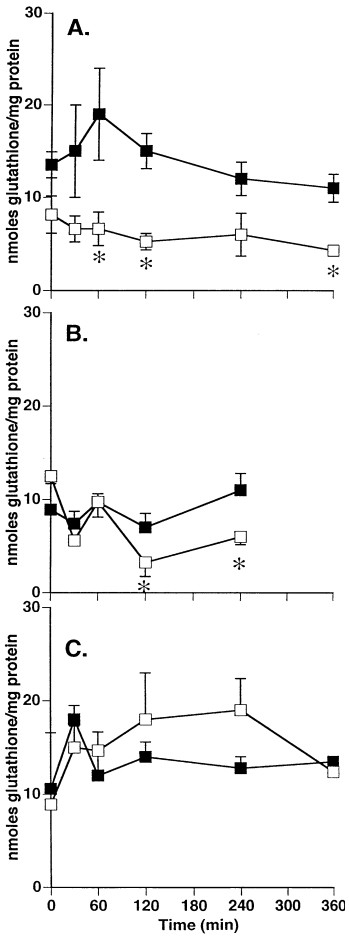

Figure 2 Intracellular glutathione content in FL5.12 cells

(**A**) Control, (**B**) *bax*- and (**C**) *bcl-x*$_L$-transfected cells (5×10^6 cells/ml) were plated in medium containing IL-3 (■) or in medium lacking IL-3 (□). After 0.5, 1, 2, 4 and 6 h, cells were collected by centrifugation and intracellular glutathione was determined as described in the Materials and methods section. Data are means ± S.E.M. ($n = 3$–6). *Significantly different from IL-3-supplemented counterparts ($P < 0.05$).

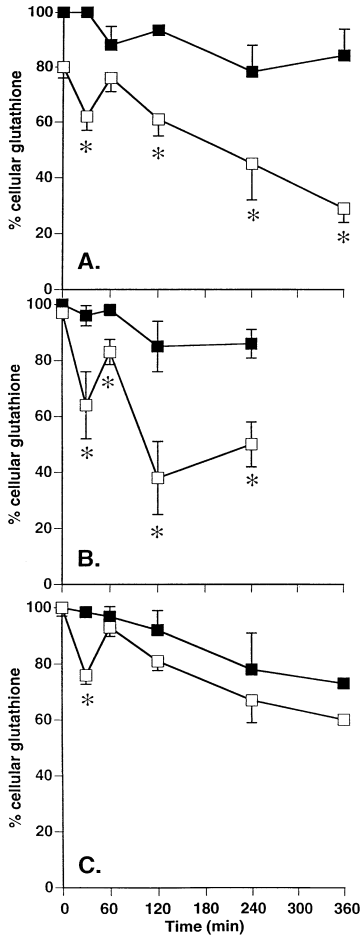

Figure 3 Glutathione efflux from FL5.12 cells

(**A**) Control, (**B**) *bax*- and (**C**) *bcl-x*$_L$-transfected cells (5×10^6 cells/ml) were plated in media containing IL-3 (■) or in media lacking IL-3 (□). After 0.5, 1, 2, 4 and 6 h, cells were collected by centrifugation. The supernatant was used to determine the glutathione levels in the medium, and the pellet was resuspended, sonicated and used to measure the intracellular content of glutathione, as described in the Materials and methods section. Data represent the mean percentage of intracellular GSH per total glutathione in both the cell lysate and the cell medium ± S.E.M. There was a small amount of GSH present in the IL-3-conditioned medium (about 2 nmol/ml) that was subtracted from the measured efflux values. *Significantly different from IL-3-supplemented counterparts ($P < 0.05$).

40% of cellular GSH occurred in FL5.12 cells within 30 min after withdrawal of IL-3. This loss was recovered in the medium in both control and *bax*-transfected cells (Figures 3a and 3b). The decrease was continuous, and at 6 h ~ 66% of the total GSH in the control cell culture flasks was found in the medium. *bax* transfectants showed a similar reduction in cellular GSH with time following IL-3 withdrawal, and at 4 h ~ 46% of total GSH was in the medium. In contrast, except for a small decline at 30 min, *bcl-x*$_L$ transfectants subjected to IL-3 deprivation did not display a significant decrease in the percentage of cellular GSH compared with control counterparts throughout the 6 h time period examined (Figure 3c).

DISCUSSION

The Bcl-2 protein can prevent oxidant-induced cell death, but it is clearly not a classical antioxidant, as it does not react directly with free radicals [31] or lipid peroxides [32]. It is possible that overexpressing Bcl-2 can prevent apoptosis without altering the production of ROS. For example, the effects of ROS can be blocked by up-regulating antioxidant enzyme activity and/or GSH levels. Analyses of enzymic antioxidant systems in control and *bcl*-transfected cell lines have not revealed any general pattern of increased activity ([33]; the present study). However, the overexpression of Bcl-2 in various cell lines has been associated with elevated levels of intracellular GSH, suggesting a role for this tripeptide in the mechanism by which Bcl-2 prevents cell death [15,33]. Because Bcl-x_L has similar anti-apoptotic ability and subcellular localization as Bcl-2, it is possible that this protein alters GSH in an analogous manner.

The present study shows that, during the 6 h incubation examined, levels of GSH do not vary between control and *bcl-x*$_L$-transfected cells in the presence of IL-3, but are significantly lower in *bax* transfectants. Following the initiation of apoptosis by growth-factor withdrawal, a dramatic decrease in GSH levels occurred in both control and *bax*-transfected cells but not in cells overexpressing *bcl-x*$_L$. This change was evidence before any alteration in apoptosis, viability of membrane permeability.

Although GSH depletion often reflects an intracellular oxidation process, GSSG did not accumulate in response to the withdrawal of IL-3. It is possible that GSSG was formed but was

rapidly exported from cells by ATP-dependent pumps, such as the glutathione S-conjugate export pump and the multispecific organic-anion transporter [34–36]. However, based on the results with 1,3-[bis-(2-chloroethyl)-1-nitrosurea, it seems more likely GSh was being eliminated by a mechanism, independent of any oxidation processes. Finding stoichiometric amounts of GSH in the media without significant changes in viability indicates that an efflux mechanism similar to that reported in JURKAT cells stimulated to undergo apoptosis by Fas/APO-1 may be operant. The ability of *bcl-$_L$* to present this efflux is the first report of this activity. Although most of the GSH exported into the media may come from the cells that most rapidly undergo apoptosis, it is just as likely that GSH is lost relatively consistently from all cells, and that it is just the rate at which apoptosis develops that varies. Overall, these results suggests that *bcl-x_L* overexpression may prevent apoptosis by maintaining intracellular GSH.

Although the exact mechanism by which *bcl-x_L* regulates the cell's redox status is not known, recent work supports a regulatory role for ions or small molecules [37]. This hypothesis is based on studies that showed that the molecular structure of Bcl-x_L resembles the structure of the pore-forming domains of several bacterial toxins, such as diphtheria toxin [38]. The critical feature of these pore-forming domains is their ability to form ion channels. By using artificial membranes, it was shown that Bcl-x_L can form an ion channel with specific ion selectivity at physiological pH for K^+ and Na^+ and, to a lesser extent, Ca^{2+} [37].

In separate studies, it was hypothesized that small proteins, such as cytochrome *c*, can be regulated by the Bcl proteins [39,40]. Cytochrome *c* is thought to initiate apoptosis by activating caspases [41,42]. Because Bcl-x_L is also located on the membranes of the nucleus and endoplasmic reticulum, sites where cytochrome *c* is not found, it is possible that Bcl-x_L regulates other molecules. One such factor can be GSH. It is logical to predict that the presence of Bcl-x_L in membranes of the mitochondria, nucleus and endoplasmic reticulum (all sites of electron transport) inhibits the translocation of GSH from these cellular organelles to an extracellular site (e.g. the media) and, thereby, prevents cell death following an apoptotic stimulus. In support of this claim, recently it was shown that CD40 cross-linking inhibited B-cell apoplasts by up-regulating *bcl-x_L* and preserving the intracellular redox state [43].

In summary, the onset of apoptosis is associated with a fall in intracellular GSH in numerous cellular systems [44–46]. The current study confirms these findings using FL5.12 cells, and demonstrates that it is not due to intracellular oxidation but rather to the translocation of GSH out of the cell and into the media. Most importantly, the loss of GSH into the media was prevented in cells overexpressing Bcl-x_L. Thus it is possible that Bcl-x_L prevents cell death by a mechanism that prevents GSH translocation.

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REFERENCES

1 Payne, C. M., Bernstein, C. and Bernstein, H. (1995) Leuk. Lymphoma **19**, 43–93
2 Thompson, C. B. (1995) Science **267**, 1456–1462
3 Corcoran, G. B., Fix, L., Jones, D. P., Moslen, M. T., Nicotera, P., Oberhammer, F. A. and Buttyan, R. (1994) Toxicol. Appl. Pharmacol. **128**, 169–181
4 Krajewski, S., Tanaka, S., Takayama, S., Schilber, M. J., Fenton, W. and Reed, J. C. (1993) Cancer Res. **53**, 4701–4714
5 Vaux, D. L., Cory, S. and Adams, J. M. (1988) Nature (London) **335**, 440–442
6 Hockenbery, D. M., Nuñez, G., Milliman, C., Schreiber, R. D. and Korsmeyer, S. J. (1990) Nature (London) **348**, 334–336
7 Chao, D. T., Linette, G. P., Boise, L. H., White, L. S., Thompson, C. B. and Korsmeyer, S. J. (1995) J. Exp. Med. **182**, 821–828
8 Gonzalez-Garcia, M., Perez-Ballestero, R., Ding, L., Duan, L., Boise, L. H., Thompson, C. B. and Nuñez, G. (1994) Development **120**, 3033–3042
9 Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nuñez, G. and Thompson, C. B. (1993) Cell **74**, 597–608
10 Oltvai, Z. N., Milliman, C. L. and Korsmeyer, S. J. (1993) Cell **74**, 609–619
11 Simonian, P. L., Grillot, D. A., Merino, R. and Nuñez, G. (1996) J. Biol. Chem. **271**, 22764–22772
12 Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Millima, C. L. and Korsmeyer, S. J. (1993) Cell **75**, 241–251
13 Vies, D. J., Sorenson, C. M., Shutter, J. R. and Korsmeyer, S. J. (1993) Cell **75**, 229–240
14 Zhong, L.-T., Sarafian, T. A., Kane, D. J., Charles, A. C., Mah, S. P., Edwards, R. H. and Bredesen, D. E. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 4533–4537
15 Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T. and Bredesen, D. E. (1992) Science **262**, 1274–1277
16 Hug, H., Enari, M. and Nagata, S. (1994) FEBS Lett. **351**, 311–313
17 Muschel, R., Bernhard, E. J., Garza, L., McKenna, W. G. and Koch, C. J. (1995) Cancer Res. **55**, 995–998.
18 Shimizu, S., Eguchi, Y., Kosaka, H., Kimiike, W., Matsuda, H. and Tsujimoto, Y. (1995) Nature (London), **374**, 811–813
19 van den Dobblesteen, D. J., Nobel, C. S. I., Schlegel, J., Cotgreave, I. A., Orrenius, S. and Slater, A. F. G. (1996) J. Biol. Chem. **271**, 15420–15427
20 Slater, A. F. G., Stefan, C., Nobel, I., van den Dobbelsteen, D. J. and Orrenius, S. (1996) Death Differ. **3**, 57–62
21 Lee, J. C., Hapel, A. J. and Ihle, J. N. (1982) J. Immunol. **128**, 2393–2398
22 Lowry, O. H., Rosebrough, N. J., Far, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
23 Duke, R. C. and Cohen, J. J. (1992) in Current Protocols in Immunology (Coligan, J. E. and Kruisbeak, A. M., eds.), pp. 3.17.1–3.17.6, John Wiley and Sons, New York
24 Mitchell, D. B., Santone, K. S. and Acosta, D. (1980) J. Tissue Culture Methods **6**, 113–115
25 Luck, H. (1965) in Methods of Enzymatic Analysis, (Bergmeyer, H. U., ed.), pp. 885–892, Verlag Chemie, Weinheim
26 Carlberg, I. and Mannervik, B. (1975) Methods Enzymol. **113**, 484–490
27 Gunzler, W. A. and Flohe, L. (1985) in CRC Handbook of Methods for Oxygen Radical Research (Greenwald, R. A., ed.), pp. 285–290, CRC Press, Boca Raton
28 Neuschwander-Tetri, B. A. and Roll, J. (1989) Anal. Biochem. **179**, 236–241
29 Liu, H. and Kehrer, J. P. (1996) Free Radicals Biol. Med. **20**, 433–442
30 Harlen, J. M., Levin, J. D., Callahan, K. S., Schwartz, B. R. and Harker, L. A. (1984) J. Clin. Invest. **73**, 706–713
31 Marshall, K., Ellerby, L., Bredesen, D. E., Aruoma, O. I. and Halliwell, B. (1995) Oxygen '95: Abstr. Meet. Oxygen Soc., p. 53
32 Sandstrom, P. A., Pardi, D., Tebbey, P. W., Dudek, R. W., Terrian, D. M., Folks, T. M. and Buttke, T. M. (1995) FEBS Lett. **265**, 66–70
33 Ellerby, L. M., Ellerbyk, H. M., Park, S. M., Holleran, A. L., Murphy, A. N., Fiskum, G., Kane, D. J., Testa, M. P., Kayalar, C. and Bredesen, D. E. (1996) J. Neurochem. **67**, 1259–1267
34 Oude Elferink, R. P. J., Ottenhoff, R., Radominska, A., Hofmann, A. F., Kuipers, F. and Jansen, P. L. M. (1991) Biochem. J. **274**, 281–286
35 Ishikawa, T. and Ali-Osman, F. (1993) J. Biol. Chem. **268**, 20116–20125
36 Ishikawa, T. (1992) Trends Pharmacol. Sci. **17**, 463–468
37 Minn, A. J., Velez, P., Schendel, S. L., Heng, L., Muchmore, S. W., Fesik, S. W., Fill, M. and Thompson, C. B. (1997) Nature (London) **385**, 353–357
38 Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S.-L. et al. (1990) Nature (London) **381**, 335–341
39 Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P. and Wang, X. (1997) Science **275**, 1129–1132
40 Kluck, R. M., Bossy-Wetzel, E., Green, D. R. and Newmeyer, D. D. (1997) Science **275**, 1132–1136
41 Liu, X., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X. (1996) Cell **86**, 147–157
42 Martin, S. J. and Green, D. R. (1995) Cell **82**, 349–352
43 Wang, F., Nath, K. A., Mackey, M. F., Noelle, R. J., Mueller, D. L. and Behrens, T. W. (1997) Am. J. Physiol. **272**, C950–C956
44 Beaver, J. P. and Waring, P. (1995) Eur. J. Cell Biol. **68**, 47–54
45 Watson, R. W. G., Rotstein, O. R., Nathens, A. B., Dackiw, A. P. B. and Marshall, J. C. (1996) Surgery **120**, 150–158
46 Bustamante, J., Tovar-B, A., Montero, G. and Boveris, A. (1997) Arch. Biochem. Biophys. **337**, 121–128

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