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Apoptosis in hematopoietic cells (FL5.12) caused by interleukin-3 withdrawal: relationship to caspase activity and the loss of glutathione

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Abstract

The mechanism of cell death caused by cytokine deprivation remains largely unknown. FL5.12 cells (a murine prolymphocytic cell line), following interleukin-3 (IL-3) withdrawal, undergo a decrease in intracellular glutathione (GSH) that precedes the onset of apoptosis. In the present study, the induction of apoptosis following IL-3 withdrawal or GSH depletion with DL-buthionine-[S,R,]-sulfoximine (BSO) was examined. Both conditions caused time-dependent increases in phosphatidylserine externalization, acridine orange and ethidium bromide staining, decreases in mitochondrial membrane potential, processing and activation of caspase-3 and proteolysis of the endogenous caspase substrate poly(adenosine diphosphate ribose)polymerase (PARP). Apoptosis induced by IL-3 deprivation but not BSO also caused lamin B_1 cleavage, suggesting activation of caspase-6. Despite a more profound depletion of GSH after BSO than withdrawal of IL-3, the extent of apoptosis was somewhat lower. Benzyloxycarbonyl-Val-Ala-Asp(OMe)fluoromethyl ketone (z-VAD.fmk) blocked this caspase activity and prevented cell death after BSO exposure but not after IL-3 deprivation. Following IL-3 withdrawal, the caspase inhibitors z-VAD.fmk and boc-asp(OMe)fluoromethylketone (boc-asp.fmk) prevented the cleavage and activation of caspase-3, the breakdown of lamin B_1 and partially mitigated PARP degradation. However, the externalization of phosphatidylserine, the fall in mitochondrial membrane potential and subsequent apoptotic cell death still occurred. These results suggest that IL-3 withdrawal may mediate cell death by a mechanism independent of both caspase activation and the accompanying loss of GSH.

Keywords: apoptosis; caspases; glutathione; interleukin-3; FL5.12 cells

Abbreviations: IL-3, interleukin-3; GSH, glutathione; BSO, DL-buthionine-[S,R]-sulfoximine; DEVD.AMC, acetyl-Asp-Glu-Val-Asp-amino methyl coumarin; PARP, poly(adenosine diphosphate ribose) polymerase; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone; boc-asp.fmk, boc-asp-(OMe)-fluoromethyl ketone; GSSG, glutathione disulfide; $DiOC_6$, 3,3′-dihexylacarbocyanine iodide

Introduction

Apoptosis is a structurally distinct form of cell death that involves a series of well-organized steps requiring active cellular participation. It is critical during development and tissue homeostasis as well as in the pathogenesis of a variety of diseases.[1,2] Apoptosis occurs in two phases; an initial commitment to cell death phase followed by an execution phase characterized by distinct structural changes.[3] These alterations include cell shrinkage, nuclear condensation, DNA fragmentation, externalization of phosphatidylserine and, finally, the breakdown of the cell into small fragments (apoptotic bodies) that in most tissues are then phagocytosed.

Increased attention has been focused on the role of a growing family of cysteine aspartate proteases (caspases) as mediators of the various changes associated with the execution phase of apoptosis.[4,5] To date, at least ten different caspase-related proteases have been identified,[6] all of which are synthesized as inactive precursors requiring cleavage at specific Asp residues to form the active enzyme.[7,8] Although caspases are responsible for the cleavage of many important cellular substrates resulting in the stereotypic morphological changes associated with apoptosis the precise ordering of these caspases in all forms of apoptosis and whether all critical ones have been identified, is not known.

Multiple lines of evidence implicate caspase-3 (CPP32/Apopain/YAMA) as a key executioner of apoptosis. For example, in many biological systems this enzyme cleaves intracellular proteins including poly(adenosine diphosphate ribose) polymerase (PARP, UI-70K and DNA-PKcs,[9–11] and its inhibition prevents apoptosis.[12] Further, caspase-3, the most closely related caspase to *ced-3*, is widely distributed and is highly expressed in cells of lymphocytic origin, suggesting that it may be an important mediator of apoptosis in the immune system.[13] Other caspases, such as caspase-7 (Mch3/ICE-LAP3) which is closely related to caspase-3, and caspase-1, can also be critical in apoptosis as they may cleave the same substrates.[6] However, because apoptosis proceeds normally in caspase-1-(interleukin converting enzyme-ICE)-deficient mice, this caspase may not be essential, and it may not be a true mammalian ced-3 counterpart.[14,15]

Apoptosis can be initiated by a variety of stimuli including reactive oxygen species (ROS).[16–18] Although disputed, several lines of evidence implicate such species as key mediators in apoptosis. For example, the formation of ROS can usually be detected upon the induction of apoptosis by a variety of means including xenobiotics, withdrawal of serum or other required growth factors and corticosteroids. Various antioxidants [including glutathione (GSH)] inhibit apoptosis induced by both oxidizing and non-oxidizing agents.[19,20] Furthermore, depleting GSH with DL-buthionine-[S,R,]-sulfoximine [BSO] or diethyl maleate induced apoptosis[21] and the diethyl maleate effect could be reversed by N-acetylcysteine. This suggested that antioxidants may provide some protection by slowing oxidative processes that occur in the absence of GSH.

Although a depletion of intracellular GSH occurs in several different cell types, including FL5.12 cells, at very early stages of apoptosis,[22–24] the intracellular oxidation usually assumed to occur following GSH loss has not always been substantiated. One explanation for this discrepancy is 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 oxidative species.[24] Attempts to enhance GSH content in these cells did not prevent apoptosis, possibly due to rapid efflux. In contrast, the inhibition of GSH efflux by bcl-2 overexpression[22] or chemically[25] is highly protective.

Taken together, these data suggest that several apoptotic pathways involve cellular thiols and thus modification of intracellular GSH content may be a key factor in apoptosis. In order to better understand the role of GSH in apoptosis FL5.12 cells were either deprived of IL-3 or depleted of GSH with BSO. Both treatments caused a time-dependent increase in apoptosis. Caspase inhibitors prevented apoptosis in BSO-treated cells suggesting a caspase-dependent mechanism, but did not block apoptotic changes or non-apoptotic decreases in viability following IL-3 withdrawal. Therefore, cell death after IL-3 deprivation is mediated by more than the loss of GSH and may involve a caspase-independent mechanism.

Results

Loss of intracellular GSH following IL-3 withdrawal and BSO exposure

The loss of GSH following IL-3 withdrawal in FL5.12 cells has been shown previously[22] and was confirmed in the present study. IL-3 deprivation caused a time-dependent decrease of intracellular GSH beginning within 3 h. GSH levels were maximally (~60%) decreased by 24 h (Figure 1). BSO treatment caused an overall greater reduction of GSH than IL-3 deprivation. Beginning at 3 h after BSO, GSH content was reduced by 70% and at 9 h GSH levels were depleted to values less than 10% of controls (Figure 1). GSH levels remained reduced for the 48 h period examined (data not shown). No changes in glutathione disulfide (GSSG) were detected in either cells deprived of IL-3 or treated with BSO (Table 1).

To investigate whether caspases were involved in the mechanism by which IL-3 deprivation and BSO administration caused GSH depletion, cells were incubated with 200 μM z-VAD.fmk, a cell permeable irreversible inhibitor of caspase-1 and caspase-3 homologs.[26–28] Although the presence of z-VAD.fmk appeared to slightly accelerate the loss of GSH following BSO, it had no effect after

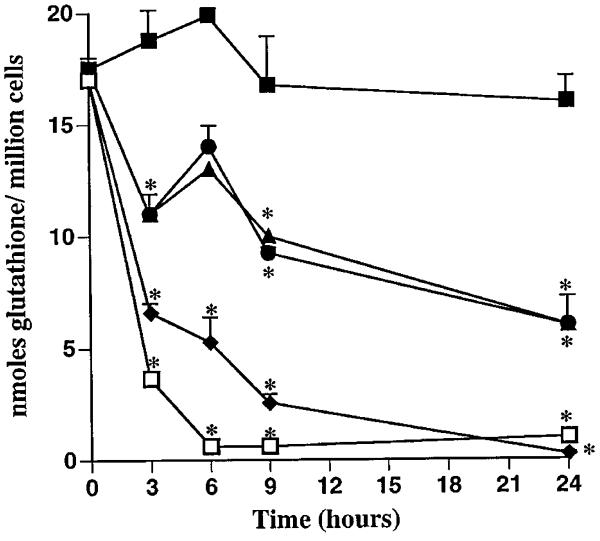

Figure 1 Loss of GSH following IL-3 withdrawal and after BSO exposure. Cells were left untreated, cultured in IL-3 free media or exposed to BSO (10 mM). The effect of z-VAD.fmk (200 μM) was examined on cells deprived of IL-3 and treated with BSO. At indicated time points, cells (1×10^6 ml^{-1}) were collected by centrifugation and GSH was derivatized with dansyl chloride prior to separation and detection with HPLC as described under Materials and Methods. Data points show mean ± S.E. (n=3–4). *Significantly different from time zero. (■) +IL-3; (●) −IL-3; (▲ −IL-3 in the presence of z-VAD.fmk; (◆) BSO; (□) BSO in the presence of z-VAD.fmk

Table 1 Cells were left untreated, cultured in IL-3 free media or exposed to BSO (10 mM).

	GSSG (nmoles/10^6cells)				
	Time (hours)				
Treatment	**0**	**3**	**6**	**9**	**24**
+IL-3	0.32 ± 0.02	0.43 ± 0.86	0.37 ± 0.05	0.36 ± 0.07	0.37 ± 0.16
−IL-3	0.32 ± 0.02	0.40 ± 0.10	0.43 ± 0.10	0.42 ± 0.12	0.30 ± 0.09
BSO	0.32 ± 0.02	0.36 ± 0.09	0.29 ± 0.13	0.24 ± 0.10	0.23 ± 0.10

At indicated time points, cells were collected by centrifugation and GSSG was derivatized with dansyl chloride prior to separation and detection with HPLC as described under Materials and Methods. Data points show mean ± S.E. (n=3–4)

withdrawing IL-3 indicating that GSH levels are not regulated by caspase-3-like proteases (Figure 1).

z-VAD.fmk blocks apoptosis induced by BSO but not by IL-3 withdrawal

A significant fourfold increase in apoptosis, as assessed by phosphatidylserine externalization on viable cells (as assessed by propidium iodide staining), was apparent at 9 h and was maximal at 20 h after IL-3 deprivation (tenfold increase over controls) (Figure 2A). These results were confirmed by examining cellular morphology using a combination of the fluorescent dyes acridine orange and ethidium bromide, which can differentiate apoptotic from necrotic cells.[29] In the presence of IL-3, the percentages of apoptotic and necrotic cells were ~5 and ~4, respectively, up to 24 h. Following IL-3 withdrawal, apoptosis increased to 17% of viable cells at 9 h, and to 31% at 24 h. Cell viability, as assessed by propidium iodide exclusion, remained at ~80% up to 20 h after IL-3 was withdrawn, but decreased significantly to 55% at 24 h (data not shown). Thus, the onset of apoptosis occurred prior to any significant decreases in overall cell viability and correlated with the loss of GSH (Figure 1).

Cells were next treated with BSO to determine whether GSH depletion could cause apoptosis similar to that observed in cells deprived of a growth factor. BSO caused an ~twofold increase in the percentage of viable cells that were apoptotic by 6 h (Figure 2B). Cell viability after BSO, as measured by propidium iodide exclusion, was ~86% (14% of the cells were necrotic) up to 30 h when it was significantly reduced to ~70% (data not shown). Thus, similar to deprivation of IL-3, BSO-induced apoptosis was evident before any significant change in overall cell viability occurred.

In contrast to cells deprived of IL-3 that exhibited a tenfold increase in apoptosis, BSO exposure only caused a fourfold induction of apoptosis at 24 h despite a greater reduction in GSH content. Because viability remained high and only a relatively small percentage of cells were apoptotic 24 h after BSO treatment, the time course was extended. Apoptosis did not significantly change up to 48 h after BSO when compared to values at 24 h. However, it should be noted that since the number of propodium iodide including-cells increased after 30 h and the percentage of apoptotic cells was still maintained within a smaller population of viable cells, it is likely that some of these apoptotic cells were undergoing secondary necrosis. These results with phosphatidylserine externalization were confirmed using acridine orange and ethidium bromide staining. Apoptosis by this method increased from basal levels of 6 to 22% and 15% at 24 and 48 h after BSO, respectively. Collectively, these results suggest that reducing GSH may not be the critical factor responsible for the increase in apoptosis observed after IL-3 withdrawal.

To investigate the role of caspases in the apoptosis caused by IL-3 withdrawal and BSO treatment, cells were incubated with z-VAD.fmk. Although this caspase inhibitor completely abrogated the increase in apoptotic cells by BSO, it did not affect the increasing number of non-viable cells and only delayed by 3 h the apoptosis induced by IL-3 withdrawal (Figure 2A and B). Administration of a second dose of z-VAD.fmk at 9 h did not prevent the

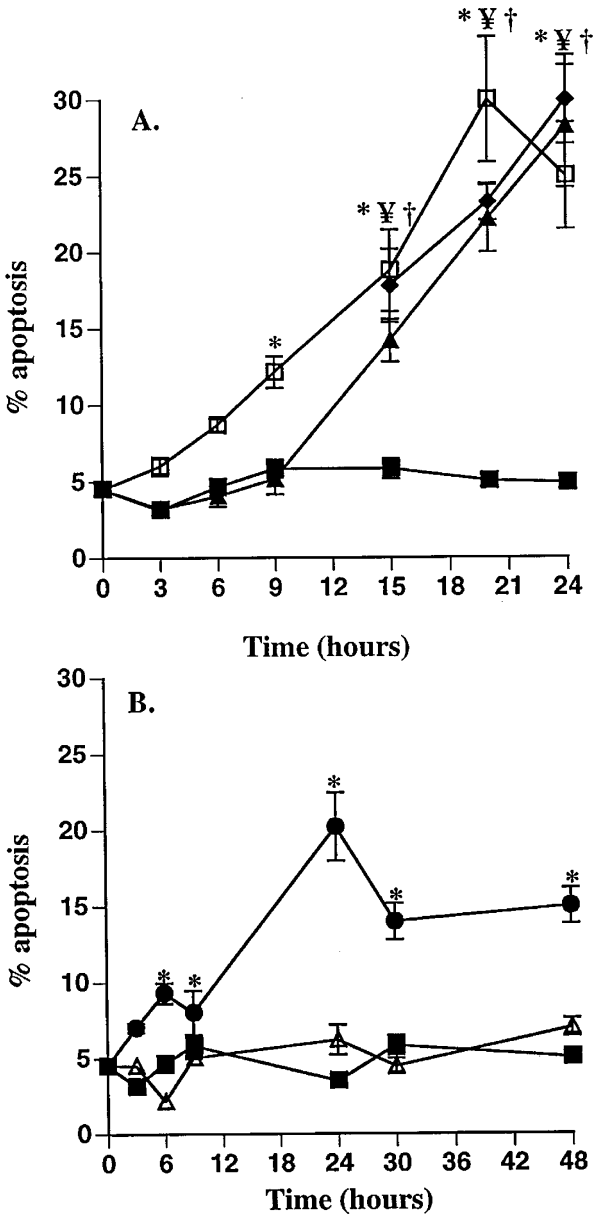

Figure 2 Caspase inhibitors block apoptosis induced by BSO but not following Il-3 withdrawal. The percentage of apoptotic cells was determined by flow cytometric analysis following FITC labelling of Annexin V binding and exclusion of propidium iodide, respectively, as described under Materials and Methods. Data points show mean ± S.E. (n=3–9) *,¥,†Significantly different from time zero for cells deprived of IL-3, deprived of IL-3 and incubated with either z-VAD.fmk or boc-asp.fmk, respectively. (**A**) Cells were left untreated or cultured in IL-3 free media. The effect of z-VAD.fmk (200 μM) or boc.asp.fmk (200 μM) was examined on cells deprived of IL-3. (**B**) Cells (1×10^6 ml^{-1}) were cultured in media supplemented with IL-3. The effect of BSO (10 mM) was examined on cells in the presence or absence of z-VAD.fmk (200 μM). (■) +IL-3; (□) −IL-3; (▲) −IL-3 in the presence of z-VAD.fmk; (◆ −IL-3 in the presence of boc-asp.fmk; (●) BSO; (△) BSO in the presence of z-VAD.fmk

subsequent increase in apoptosis (data not shown) indicating that the failure to prevent apoptosis was not due to a limited supply of inhibitor.

The effect of boc-asp.fmk, a broad spectrum caspase inhibitor,[30] on cells deprived of IL-3 was examined at 15, 20 and 24 h, time points that displayed large increases in apoptosis. Similar to z-VAD.fmk, boc-asp.fmk was unable to mitigate the increase in apoptosis induced by withdrawal of IL-3 (Figure 2A). Results obtained using acridine orange and ethidium bromide were similar (data not shown). These results suggest that the apoptosis that occurs upon withdrawal of IL-3 does not require caspase activity.

Caspase inhibitors do not prevent the fall in mitochondrial membrane potential following IL-3 withdrawal and BSO treatment

In several models of apoptosis, a decrease in mitochondrial membrane potential precedes nuclear apoptotic changes.[31–33] The number of cells with decreased mitochondrial membrane potential became significant 15 h after withdrawal of IL-3 and included ~20% of all cells within 24 h (Figure 3A). Neither z-VAD.fmk nor boc-asp.fmk could prevent this decrease, suggesting that either changes in mitochondrial membrane potential following withdrawal of IL-3 lie upstream of caspase activation or that caspases are not involved in this process. Interestingly, at 24 h the presence of caspase inhibitors seemed to increase the number of IL-3 deprived cells with reduced mitochondrial membrane potential. The significance of this effect is not known.

In contrast to withdrawing IL-3, mitochondrial membrane potential was maintained with BSO exposure until 48 h when ~14% of cells demonstrated a decline (Figure 3B). Interestingly, the onset of apoptosis occurred prior to the drop in mitochondrial membrane potential, suggesting this change is not essential for apoptosis.

Processing of caspase-3 accompanies the induction of apoptosis caused by IL-3 withdrawal and BSO treatment

Western blot analyses were performed using antibodies to the p17 fragment of caspase-3 to determine whether time-dependent processing of caspase-3 occurred following IL-3 withdrawal and after BSO exposure. In untreated cells, immunoblots showed the presence of the 32-kD precursor of caspase-3 (Figure 4A). After withdrawal of IL-3, the p17 subunit of the mature caspase-3 enzyme was detected at 9 h and increased relative to the 32-kD precursor to 24 h (Figure 4B). Concurrently, a time-dependent decrease in the level of the 32-kD precursor of caspase-3 occurred. After BSO exposure a time-dependent increase in the 17-kD fragment was evident at 9 h, although no decreases in the 32-kD precursor were noted (Figure 4C). The caspase inhibitor z-VAD.fmk completely blocked the loss of the pro-caspase-3 and the formation of the p17 subunit following both IL-3 deprivation and BSO exposure (Figure 4A and B).

PARP and lamin B_1 degradation following IL-3 withdrawal and BSO treatment

The intact form of PARP (116-kD) (Figure 4D) and lamin B_1 (66-kD) (Figure 4E) was detected in the presence of IL-3. After IL-3 withdrawal, 116-kD PARP and 66-kD lamin B_1 decreased with time and within 24 h the proforms were dramatically reduced (Figure 4D and E). Coincident to

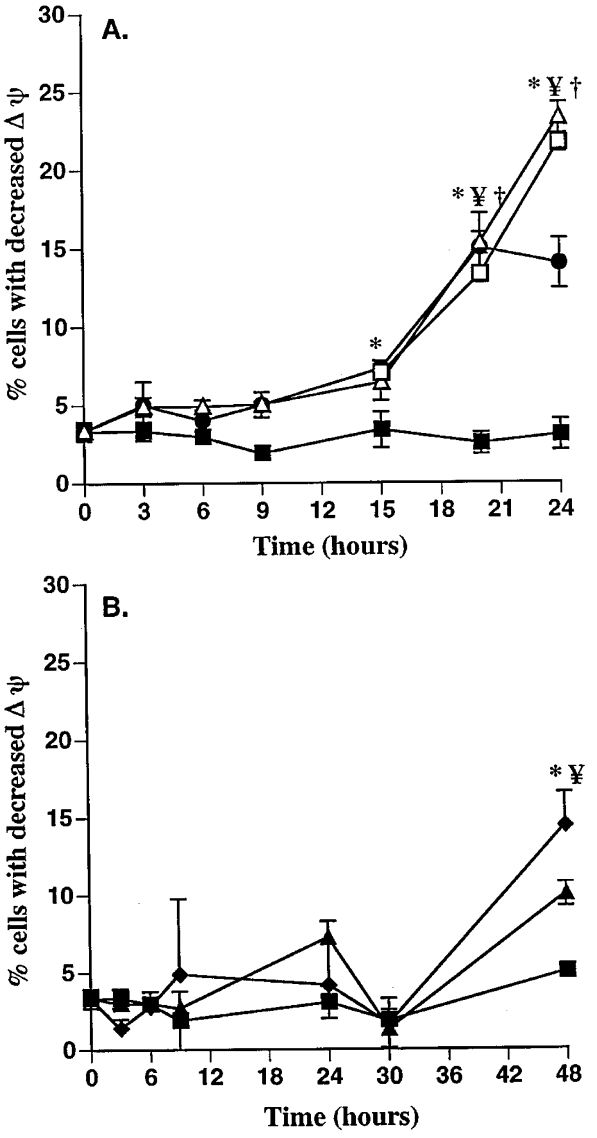

Figure 3 IL-3 deprivation and BSO exposure decreases the mitochondrial membrane potential. The percentage of cells with decreased mitochondrial membrane potential was determined by flow cytometric analysis as described under Materials and Methods. Data points show mean ± S.E. (n=3–9). *,¥,†Significantly different from time zero for cells deprived of IL-3, deprived of IL-3 and incubated with either z-VAD.fmk or boc-asp.fmk, respectively. (**A**) Cells were left untreated or cultured in IL-3 free media. The effect of z-VAD.fmk (200 μM) or boc.asp.fmk (200 μM) was examined on cells deprived of IL-3. (**B**) Cells were cultured in media supplemented with IL-3. The effect of BSO (10 mM) was examined on cells in the presence or absence of z-VAD.fmk. (■) +IL-3; (●) −IL-3; (□) −IL-3 in the presence of z-VAD.fmk; (△) −IL-3 in the presence of boc-asp.fmk; (◆) BSO; (▲) BSO in the presence of z-VAD.fmk

the disappearance of the proforms was the appearance of the 85-kD and 43-kD fragments of PARP and lamin B_1, respectively. Therefore, several target proteins are cleaved in the execution phase of apoptosis following IL-3 withdrawal. Cleavage of PARP suggests activation of caspase-3 and -7[12,34] and breakdown of lamin B_1 suggests activation of caspase-6.[35,36]

Although z-VAD.fmk, an inhibitor with greater specificity for caspase-3 than boc-asp.fmk, abrogated the formation of the 85-kD and 43-kD fragments of PARP and lamin B_1, respectively, it did not completely protect against the degradation of the 116-kD PARP that occurred after withdrawal of IL-3 (Figure 4D and E). In contrast, the broad spectrum caspase inhibitor boc-asp.fmk was unable to prevent the breakdown of PARP or lamin B_1 at 24 h (Figure 4D and E). Taken together these results suggest that IL-3 withdrawal activates a caspase and/or protease that targets PARP and is at least partially resistant to the caspase inhibitors tested.

In cells exposed to BSO for 24 h, a large decrease in 116-kD PARP and a small increase in the 85-kD fragment occur similar to the changes seen after withdrawal of IL-3 (Figure 4F). The degradation of PARP following BSO treatment was prevented by either z-VAD-fmk or boc-asp.fmk. However, unlike cells deprived of IL-3, cells exposed to BSO did not demonstrate any breakdown of lamin B_1 (Figure 4G). Because z-VAD.fmk prevented apoptosis after BSO, together these results suggest that the apoptosis caused by BSO is mediated by caspases.

IL-3 withdrawal and BSO exposure activate DEVD.amc cleavage in cell lysates

The activation of a caspase activity is considered an important effector of apoptosis. In order to examine whether growth factor withdrawal and BSO treatment induced either casapase-3 or -1-like activities, the cleavage of the fluorogenic peptide substrates YVAD.amc

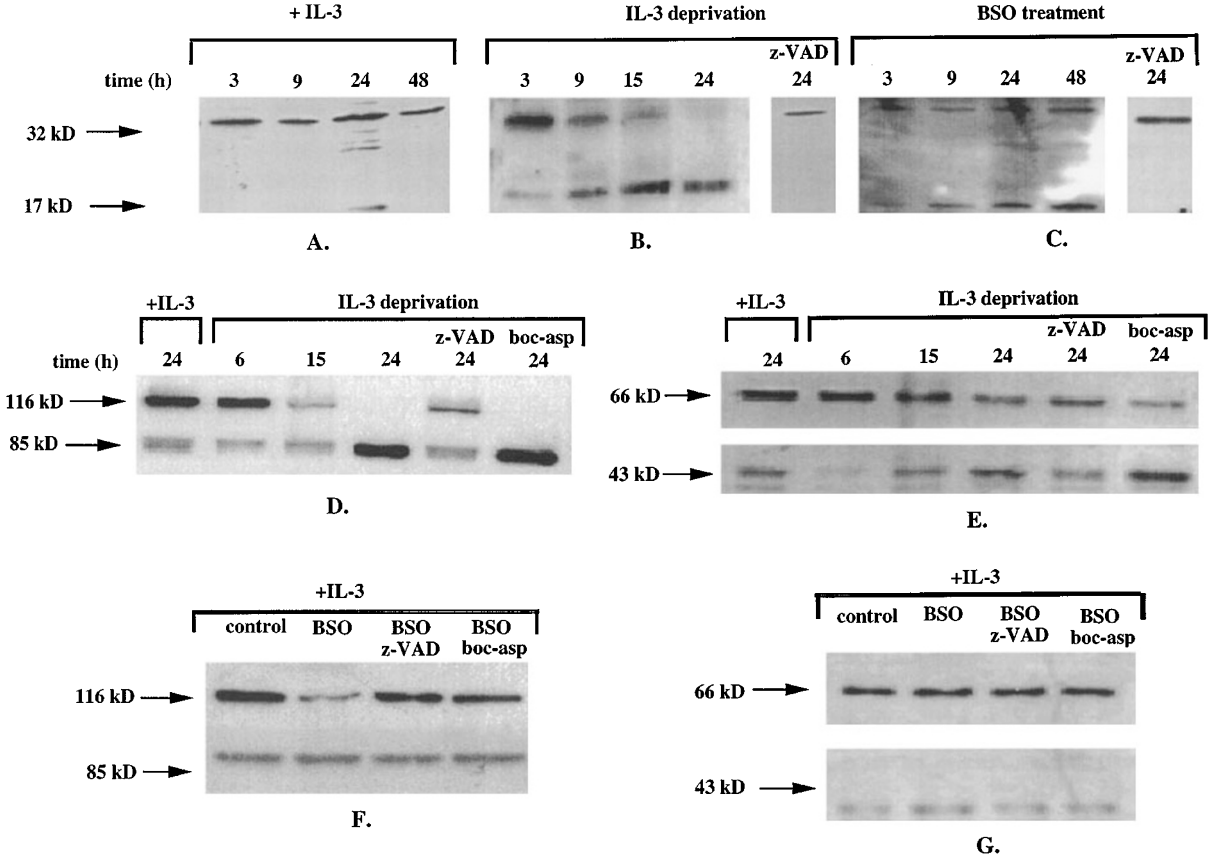

Figure 4 Cleavage of caspase-3, PARP and lamin B_1 following IL-3 withdrawal and BSO treatment. Caspase 3 cleavage was assessed in untreated cells (**A**), cells cultured in IL-3 free media (**B**) or cells exposed to BSO (10 mM) in the presence of IL-3 (**C**). The effect of z-VAD.fmk (200 μM) was examined on cells deprived of IL-3 and treated with BSO. At indicated time points, cells were collected and protein were resolved on a SDS-15% polyacrylamide gel. Cells were analyzed using antibodies to caspase-3 as described under Materials and Methods. The proform of caspase-3 (32-kD) and cleaved proteolytic fragment (17-kD) are indicated by the upper and lower arrows, respectively. PARP (**D**, **F**) and lamin B_1 (**E**, **G**) degradation were assessed in untreated cells or cells cultured in IL-3 free media or treated with BSO (10 mM) for 24 h. The effect of z-VAD.fmk (200 μM) or boc-asp.fmk (200 μM) was examined at 24 h. At the indicated times, cells were collected and proteins were resolved on a SDS-8% polyacrylamide gel. Cells were analyzed using antibodies to PARP and lamin B_1 as described under Materials and Methods. The proform of PARP (116-kD) and cleaved proteolytic fragment (85-kD), and the proform of lamin B_1 (66-kD) and cleaved fragment (43-kD) are indicated by the upper and lower arrows, respectively

and DEVD.amc was measured. The DEVD.amc cleaving activity of lysates obtained from control cells was very low and did not increase during the time course studied (Figure 5A and B). In contrast, enzymatic cleavage of DEVD.amc was detected 9 h after IL-3 withdrawal and 30 h after BSO exposure and increased thereafter (Figure 5A and B). The addition of Ac.DEVD.cho, a caspase inhibitor relatively specific for caspases-3 and -7, or z-VAD.fmk completely blocked caspase-3-like activity in cells deprived of IL-3 or treated with BSO. In contrast, no cleavage of YVAD.amc was detected using either apoptotic stimuli (data not shown). Taken together, these results suggest that caspase-3-like but not caspase-1-like proteases are activated in apoptosis mediated by IL-3 withdrawal and BSO.

Discussion

Apoptosis in IL-3 dependent cells

Many hematopoietic cells are dependent on cytokines for growth and survival. Although the mechanism by which cytokine withdrawal causes apoptosis is thought to involve inactivation of signaling pathways, a role for ROS, GSH and caspases in this process has also been suggested.[37,38] Caspases have attracted particular attention because of their similarity to the ced-3 'death' gene, and the ability of various inhibitors of these enzymes to block apoptosis induced by a variety of treatments.

Caspase-independent apoptosis?

Although the activation of several caspases clearly accompanied IL-3 withdrawal-induced apoptosis, neither caspase inhibitor tested could prevent apoptosis or the decrease in mitochrondrial membrane potential. The failure of an inhibitor with a broad spectrum of activity, such as boc-asp.fmk, to have an effect on apoptosis argues against a role for caspases. It remains possible, however, that caspase-3 may participate in the apoptotic process along with other caspases that are not effectively inhibited with z-VAD.fmk. The inability of either caspase inhibitor used to completely block the breakdown of PARP and lamin B_1 suggests the presence of significant residual caspase activity that may mediate apoptosis in this system. Alternately, it is possible that other proteases are the agents that mediate the execution stage of apoptosis after IL-3 withdrawal.

Several recent studies using alternate approaches have demonstrated the potential for caspase-independent apoptosis.[39–41] For example, bax induced cell death in JURKAT cells was not prevented by various inhibitors of proteases.[39] Also demonstrated was a fall in mitochondrial membrane potential and an increase in ROS, implicating a role for mitochondria, possibly due to the opening of mitochondrial pores which release soluble pro-apoptotic factors such as cytochrome *c*.[42]

Recently, it was shown that bcl-2 overexpression but not z-VAD.fmk prevented decreases in mitochondrial membrane potential and changes in plasma membrane integrity. This suggested that the activation of apoptogenic proteases occurs after changes in mitochondrial permeability.[43–45] Consistent with these observations, a fall in mitochondrial membrane potential that was not blocked by caspase inhibitors was noted in the present study as an early event after IL-3 withdrawal. In contrast, cells treated with BSO only demonstrated significant changes in mitochondrial membrane potential at 48 h; well after the onset of apoptosis. These results suggest that apoptosis caused by BSO occurs independently of

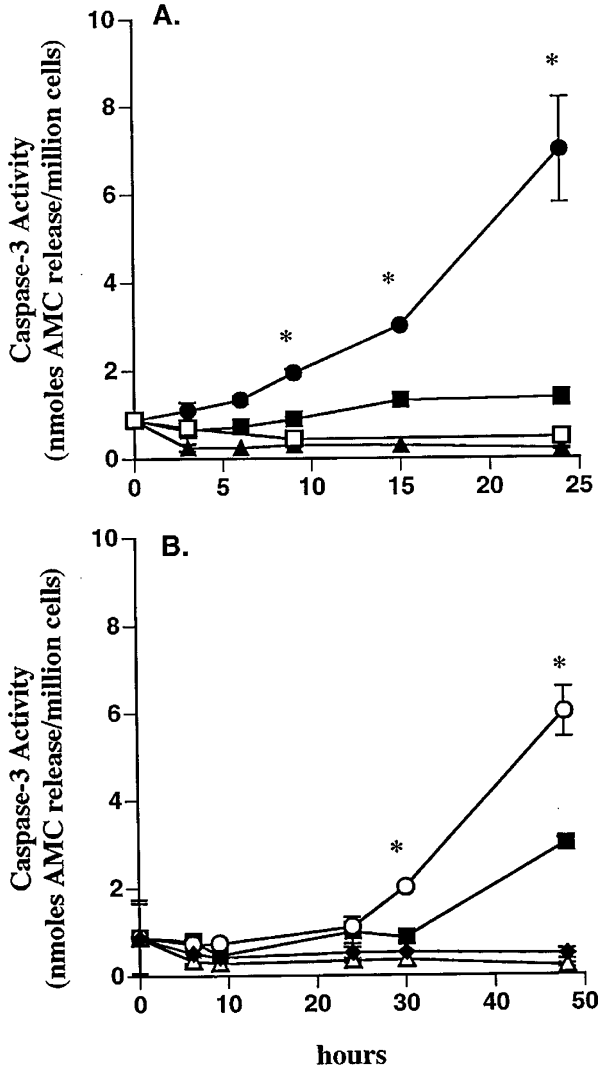

Figure 5 Cytosolic caspase-3 activity increases after IL-3 withdrawal and BSO treatment. Caspase-3-like activity was measured fluorometrically by its ability to release amc from the fluorogenic substrate Ac-DEVD.amc. Some cells were preincubated with caspase-3 inhibitor Ac-DEVD.cho (50 μM) or z-VAD.fmk (200 μM). Data represent $\pm$S.E. (n=3). *Significantly different from time zero. (**A**) Cells were left untreated or cultured in IL-3 free media. (**B**) Cells were cultured in media supplemented with IL-3. (■) +IL-3; (●) −IL-3; (▲) −IL-3 in the presence of Ac-DEVD.cho; (□) −IL-3 in the presence of z-VAD.fmk; (○) BSO; (△) BSO in the presence of z-VAD.fmk; (◆) BSO in the presence of Ac-DEVD.cho

the changes in mitochondrial membrane potential but does not necessarily exclude a role for the mitochondria in this cell death system.

Cells treated with BSO to achieve levels of GSH comparable to those attained after cytokine deprivation appeared to undergo apoptosis in a caspase-dependent manner. This suggests that caspase activation may vary with different stimuli within similar as well as different cellular lineages. For example, apoptosis induced by IL-2 removal in T cells is mediated independently of caspases-1 or -3, although an unknown caspase member may be involved.[38,46] Others have shown that caspases such as caspase-8 (FLICE) are recruited to death receptor complexes such as CD95 (Fas/Apo 1) and play a proximal role in apoptosis.[47,48] In general, while caspases are activated in most if not all apoptotic systems, and may mediate critical steps, their absolute requirement is unclear. It is possible that caspases are responsible for the proteolysis of many cellular substrates, which results in the stereotypic morphological and biochemical changes associated with apoptosis. Inhibition of the caspases may result in inhibition of these sterotypic changes but the cell is already committed to die. Thus, cell death still ensues but without the normal changes associated with apoptosis.

Mechanism of apoptosis after GSH depletion

Although the present study confirms that GSH depletion can induce apoptosis,[21,23] the data suggest that this may not play a critical, or at least direct, role in apoptosis caused by cytokine deprivation. One factor that may account for the discrepancy between cytokine deprivation and BSO-induced apoptosis is that BSO only depletes cytosolic GSH, thereby preserving GSH levels in the mitochondria as well as the nucleus that may be the critical pools to prevent apoptosis. Recently, it was shown that bcl-2 overexpression causes redistribution of glutathione to the nucleus where it alters nuclear redox status and blocks caspase activity.[49] This concept would explain the higher levels of GSH seen in cells that overexpress antiapoptotic bcl proteins.[22,50,51]

Despite the above evidence, the relationship between GSH and apoptosis remains unclear. BSO does not increase the susceptibility to radiation-induced apoptosis,[51] suggesting that GSH does not directly regulate this process. It is possible that other cellular thiols such as thioredoxin may play a role.[52] Others have shown an increase in apoptosis following diethyl maleate and diamide treatment. The effect of diamide, which causes a more general oxidation of cellular thiols subsequent to oxidation of GSH, suggests that a shift in the redox state is important for apoptosis. Although GSSG was unchanged in the current study, the drop in GSH did change the redox balance; an effect that could promote apoptosis.

Because caspases contain critical thiol sites, modification of these residues by GSH may occur and regulate caspase activity. For example, H_2O_2 can delay apoptosis as well as prevent fas-induced apoptosis by directly inhibiting the cysteine residues on caspases.[53] It is possible that GSH would keep caspases functional and primed by maintaining them in a reduced state. In support of this concept, Nicholson *et al*.[12] showed that reducing agents maintained the activity of recombinant caspase-3. These results may also explain some of the discrepancies that are observed with GSH and apoptosis and also points out that regulation of apoptosis may be a subtle balance between the level of ROS and GSH and/or overall reducing equivalents in the cell.

In summary, these data show that both deprivation of IL-3 and treatment with BSO causes a loss of GSH, activates caspases and induces apoptosis in FL5.12 cells. The addition of caspase inhibitors prevented apoptosis in BSO-treated cells but had only minimal effects following IL-3 withdrawal. These results suggest that cell death after IL-3 deprivation occurs by a caspase-independent mechanism as well as independently of the loss of GSH.

Materials and Methods

Materials

RPMI-1640 media was purchased from Gibco (Paisley, UK). Benzyloxycarbonyl-Val-Ala-Asp(OMe)fluoromethyl ketone (z-VAD.fmk) and boc-asp(OMe)fluoromethyl ketone (boc.asp.fmk) were obtained from Enzyme Systems, Inc (Dublin, CA, USA). The tetrapeptide caspase inhibitors, Acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD.cho) and acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD.cho) were purchased from Calbiochem-Novabiochem Corp (LaJolla, CA, USA). The tetrapeptide caspase substrates, Acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD.amc) and acetyl-Tyr-Val-Ala-Asp-7-amino-4-methyl coumarin (Ac-YVAD.amc), were acquired from Research Biochemicals Internationals (Natick, MA, USA). Annexin V-FITC kit was purchased from Bender MedSystems (Vienna, Austria). All other chemicals were from Sigma Chemical Co. (Poole, UK or St. Louis, MO, USA).

Cell culture

FL5.12 cells (an IL-3 dependent murine prolymphocytic cell line) were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 10% (v/v) WEHI-3B-conditioned medium, and 1% glutamine in a 5% CO_2 : 95% air atmosphere at 37°C. WEHI-3 cells were grown as described previously.[54] Cultures were maintained in logarithmic growth phase by routine passage every 2–3 days. The peptide caspase inhibitors were prepared as stock solutions in DMSO, and aliquots were added to the cultures keeping the DMSO at $\leqslant 0.1\%$ (final concentration).

Analysis of apoptosis and cell viability

Apoptosis was assessed by measuring the binding of FITC labeled Annexin V protein to the phospholipid phosphatidylserine which is present on the external surface of the membrane of apoptotic cells.[55,56] Cells (1×10^6 ml^{-1}) were incubated with FITC labeled Annexin V following the manufacturer's instruction. Non viable cells were assessed simultaneously by addition of propidium iodide.

Analyses were conducted using a Becton Dickinson FACScan flow cytometer. After excluding non-viable cells (indicated by propodium iodide uptake and DNA binding), the remaining viable cells were displayed as a cytogram of appropriate fluorescence *versus* forward light scatter. Two populations of viable cells were obtained; normal and apoptotic cells with low and high FITC-staining, respectively.

Apoptosis was also assessed by fluorescence microscopy by mixing 2 μl of acridine orange (100 μg/ml), 2 ml ethidium bromide (100 μg/ml) and 20 μl of the cell suspension. A minimum of 200 cells were differentiated from 'dead' apoptotic, necrotic and normal cells by examining the changes in cellular morphology based on distinctive nuclear and cytoplasmic fluorescence.[29]

Western blot analysis

Cells (2.5×10^6 ml^{-1}) were collected by centrifugation at $400 \times g$ for 5 min and washed twice with cold phosphate-buffered saline, pH 7.4 (PBS). Proteins were resolved on either 8% (PARP and lamin B_1) or 15% (caspase-3) SDS polyacrylamide gels and blotted onto nitrocellulose (Hybond-C extra: Amersham, Little Chalfont, UK). Intact PARP (116-kD) and its apoptotic signature fragment (85-kD) were detected with a monoclonal antibody (a gift from Dr. G. Poirier, Laval University, Quebec, Canada). A mouse monoclonal antibody was used to detect both the intact form (66-kD) and cleaved product (43-kD) of lamin B_1 (Serotec, Oxford, UK). A goat polyclonal antibody was used to detect both the pro-caspase-3 (32-kD) and the p17 subunit (Santa Cruz, CA, USA). Detection was achieved using the appropriate secondary antibody (goat anti-mouse IgG or rabbit anti-goat IgG) conjugated horse-radish peroxidase and by enhanced chemiluminescence (Amersham, Little Chalfont, UK).

Glutathione determination

Glutathione was quantitated using a method previously described.[57] Cells (1×10^6 ml^{-1}) were washed twice and precipiated with 200 μL of 5% perchloric acid (v/v), 2 mM diethylenetriamincepentaacetic acid (DEPA), and 0.2 mM boric acid. Protein was removed by centrifugation. After having alkylated free thiols with iodoacetic acid, the primary amines in the supernatant were derivatized with dansyl chloride. The derivatives of GSH and GSSG were then separated by HPLC and assessed fluorimetrically.

Quantitation of mitochondrial transmembrane potential

Cells (1×10^6 ml^{-1}) were incubated for 20 min at 37°C with 3,3′-dihexylacarbocyanine iodide ($DiOC_6$; 50 nM, Molecular Probes Inc., Eugene, OR, USA), which is retained in mitochondria with normal membrane potential.[32] Cells were then incubated for 2 min with propidium iodide (50 μg/ml) followed by analysis on a Becton Dickinson FACScan flow cytometer. Forward and side scatters were gated on the major population of normal-sized cells. Non-viable cells (propidium iodide positive) were then excluded and the remaining viable cells were displayed as a cytogram of appropriate fluorescence *versus* forward light scatter. Two populations of viable cells were obtained, cells with high and low staining for $DiOC_6$. In control experiments cells were incubated with carbonylcyanide *m*-chlorophenylhydrozone (50 μM; 10 min; 37°C), an uncoupling agent that abolishes the mitochondrial membrane potential.

Measurement of caspases-3 and -1-like activities

Caspases-3 and -1-like activities were determined following standard procedures.[7,12,58] Specifically, cells (0.7×10^6 ml^{-1}) were collected and resuspended in 300 μl of 0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl and 50 mM Tris, pH 7,5. Aliquots (50 μl) of the extracts were incubated with 40 μM tetrapeptide substrate (Ac-DEVD.amc or Ac-YVAD.amc), 10 mM HEPES (pH 7.5), 0.05 M NaCl and 2.5 mM DTT in a final volume of 200 μl for 2 h at 37°C. The fluorescence of the released 7-amino-4-methyl coumarin (amc) was measured using an excitation wave length of 365 nm and emission wave length of 450 nm.

Statistics

Data are expressed as mean $\pm$ S.E. Comparison between groups were done with a one-way analysis of variance followed by Student–Newman–Kuel's test. A P value of less than 0.05 was considered to be significant.

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