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Caspase-9 is activated in a cytochrome *c*-independent manner early during TNFα-induced apoptosis in murine cells

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Abstract

FL5.12 pro-B lymphoma cells utilize the mitochondrial pathway to apoptosis in response to tumor necrosis factor (TNF) receptor occupation, yet high levels of the Bcl-2 family antiapoptotic protein, Bcl-x_L, fail to protect these cells against TNF-receptor-activated death. Bcl-x_L expression delays, but does not totally block, the release of mitochondrial cytochrome *c* (cyt *c*) in these cells in response to TNFα-induced apoptosis and caspase-9 is processed prior to mitochondrial cyt *c* release under these circumstances. Early processing of caspase-9 also occurred in Apaf-1 knockout murine fibroblasts in response to TNF-receptor occupation. A caspase-9-specific inhibitor was more effective in delaying the progression of apoptosis in the FL5.12 Bcl-x_L cells than was an inhibitor specific to caspase-3. Furthermore, downregulation of caspase-9 levels by RNA interference resulted in partial protection of these cells against TNF-receptor-activated apoptosis, indicating that caspase-9 activation contributed to early amplification of the caspase cascade. Consistent with this, proteolytic processing of caspase-9 was observed prior to processing by caspase-3, suggesting that caspase-3 was not responsible for early caspase-9 activation. We show that murine caspase-9 is efficiently processed by active caspase-8 at SEPD, the motif at which caspase-9 autoprocesses following its recruitment to the apoptosome. Our results suggest that, in addition to processing procaspase-3 and the BH3 protein Bid, active caspase-8 can cleave and activate procaspase-9 in response to TNF receptor crosslinking in murine cells.
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Keywords: apoptosis; caspase-9; caspase-8; cytochrome *c* release; death receptors

Abbreviations: TNF-α, tumor necrosis factor-alpha; CHX, cycloheximide; cyt *c*, cytochrome *c*; DISC, death-inducing signaling complex

Introduction

Apoptosis is a tightly regulated process important for differentiation, for the regulation of cell numbers, and for the removal of aged, damaged and autoreactive cells.[1,2] A variety of extracellular and intracellular signals can trigger an apoptotic response, including growth factor deprivation, overexpression of oncogenes and tumor suppressor genes, radiation, chemotherapeutic drugs, and crosslinking of receptors such as the Fas or tumor necrosis factor (TNF) receptor. Apoptotic triggers activate intracellular response pathways that lead to the controlled activation of cysteine proteases known as caspases.[3] Two major pathways of caspase activation have been described – one involving the release of multiple polypeptides from mitochondria as a result of its destabilization and the other involving cell surface 'death' receptor activation by ligand binding.[4]

Human cells have been classified as type I or type II based on their responsiveness to activation of the TNF family of death receptors, particularly Fas ligand-mediated death.[5] Ligand binding causes trimerization of these receptors on the cell surface and recruitment of cytoplasmic adaptor proteins. These, in turn, recruit procaspase-8 molecules that self-process into their active forms.[6,7] Once activated, caspase-8 can trigger two distinct pathways of apoptosis. In type I cells, the release of large amounts of activated caspase-8 from the death-inducing signaling complex (DISC), enables direct activation of downstream caspases such as caspase-3, leading to cell death. In contrast, in type II cells DISC formation is greatly reduced, but the small amounts of active caspase-8 molecules that are generated are sufficient to induce mitochondrial apoptogenic activity. Caspase-8 cleaves the Bcl-2 family protein, Bid, to generate a C-terminal fragment that then translocates to the mitochondria causing its disruption, and triggering the release of cytochrome *c* (cyt *c*).[8-10] A cytoplasmic multiprotein complex, comprising Apaf-1, cyt *c* and the 'initiator' caspase, caspase-9, then activates a series of 'effector' caspases beginning with caspase-3 and culminating in the death of the cell.[11,12] In cells of type II, such as Jurkat, the proapoptotic effects of activated Bid on the mitochondria can be effectively inhibited in the presence of high levels of Bcl-2 or Bcl-x_L proteins.[5,13] Murine hepatocytes appear to exhibit properties of type II cells,[14] but some hematopoietic cell lines of murine origin have eluded categorization as type I or type II in terms of their responsiveness to Fas and/or TNF-receptor activation. For instance, IL-3-dependent murine pro-B FL5.12 cells recruit small amounts of procaspase-8 to the DISC and clearly utilize Bid cleavage as the primary mechanism of amplification[15] (AK, unpublished). Despite this, however, overexpressed Bcl-x_L cannot protect FL5.12 cells against TNF-receptor-mediated death. It was of interest, therefore, to understand how amplification of the apoptotic process occurred in these and

other murine cell types in response to TNF-receptor cross-linking.

Release of mitochondrial cyt *c* into the cytoplasm and its subsequent association with the Apaf-1 protein is thought to be an absolute requirement for the activation of caspase-9, the apical caspase in the mitochondrial pathway of apoptosis.[12] In the presence of ATP/dATP, cyt *c* that has been released into the cytosol binds to and triggers the oligomerization of the cytosolic Apaf-1 protein. The resultant complex recruits multiple copies of procaspase-9 leading to its activation.[12] Although autoprocessing occurs rapidly, unprocessed forms of caspase-9 are also catalytically active as part of the caspase-9–Apaf-1 holoenzyme complex.[16,17] A purified truncated form of the Apaf-1 protein lacking the WD-repeats and cyt *c* binding site was shown *in vitro* to be constitutively active in its ability to induce self-processing of procaspase-9.[18] However, two recent studies have indicated that caspase-9 can also be activated by mechanisms involving neither cyt *c* release nor Apaf-1 activation.[19,20] One study showed that, in response to endoplasmic reticular (ER) stress, caspase-12 was able to process and activate caspase-9, while another indicated that virus infection could trigger a novel, yet unknown, pathway of caspase-9 cleavage.

The primary objective of the present study was to determine the basis for the inability of overexpressed Bcl-x_L to protect FL5.12 pro-B cells from launching a rapid apoptotic response to TNF-α/cycloheximide (CHX) treatment, when the mitochondrial pathway (via Bid cleavage) and cyt *c* release appeared to be the preferred route to apoptotic death in untransfected and vector-transfected controls.

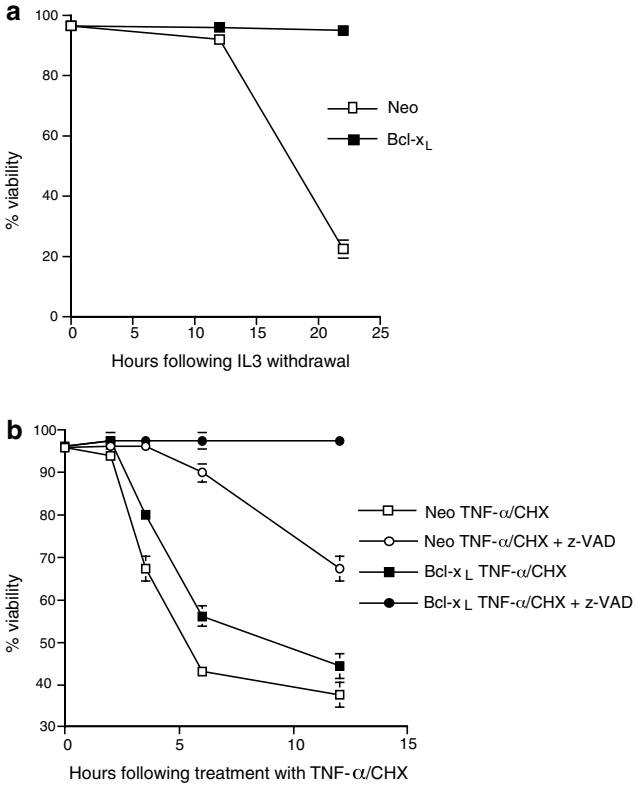


Figure 1 Bcl-x_L protects murine FL5.12 pro-B cells against growth factor withdrawal, not against death receptor activation. FL5.12 cells transfected with either pSFFV–Bcl-x_L or with the empty vector, pSFFV–Neo, were cultured in IL-3-free medium for 22 h (**a**) or incubated with TNF-α (5 ng/ml) and CHX (20 μg/ml) in the presence or absence of 50 μM of the caspase inhibitor z-VAD-fmk for 12 h (**b**). Viability (mean and standard deviations, n=3) of the transfected clones under conditions of IL3 deprivation was determined by PI exclusion and FACS analysis at the indicated time points

Results

Bcl-x_L does not protect murine pro-B cells from TNFα-induced death

Interleukin-3 (IL-3)-dependent murine FL5.12 pro-B lymphoma cells expressing high levels of transfected Bcl-x_L or the empty Neo vector were either subjected to a growth factor withdrawal assay or treated with TNF-α in the presence of CHX (described in Materials and Methods). Figure 1a and b shows that FL5.12 cells overexpressing Bcl-x_L are protected against growth factor withdrawal-induced death, as assayed by propidium iodide (PI) exclusion and flow cytometry, for as long as 96 h (22-h experiment shown in Figure 1a), but not against apoptosis caused by treatment with TNF-α and CHX. However, TNF-α/CHX-induced death in both control and Bcl-x_L cells could be inhibited in the presence of a broad-spectrum caspase inhibitor, z-VAD-fmk. The inhibition in control cells was manifest as a delayed death response, while the Bcl-x_L cells exhibited almost total resistance to apoptosis for at least 24 h (a 12-h experiment is shown in Figure 1b).

Bcl-x_L delays the release of mitochondrial cyt *c* in response to TNF-receptor activation

Since Bcl-x_L has been shown to be effective at maintaining mitochondrial integrity and preventing cyt *c* release during growth factor withdrawal,[21] we determined the cellular distribution of cyt *c* in control and Bcl-x_L-expressing FL5.12 cells exposed to TNF-α/CHX for a few hours. The Western blot in Figure 2a (top panel) shows that 3 h after induction, when apoptotic cells are easily detectable by flow cytometry (Figure 1b), mitochondria from Bcl-x_L-expressing FL5.12 cells still retain cyt *c*. Shown in the center and bottom panels are cytochrome oxidase subunit IV (COX IV) and actin Western blots, controls for the mitochondrial and cytosolic fractions, respectively. Bcl-x_L protein levels, as detected by Western blotting, did not decrease for at least 4 h following treatment with either TNF-α/CHX or CHX alone (not shown). The cyt *c* release observed in the FL5.12 Neo cells is the result of caspase-8-mediated cleavage and activation of the Bcl-2 family BH3 protein, Bid, in response to TNF-receptor cross-linking.[15] We were able to confirm the cleavage of Bid in death receptor-activated control and Bcl-x_L-expressing cells by Western blotting using antibodies against both full-length and cleaved mouse Bid protein (not shown). However, while Johnson *et al.*[15] demonstrate no release of cyt *c* from mitochondria of FL5.12 Bcl-x_L cells as late as 6 h after induction of apoptosis, our data consistently show 30–50% loss of the protein from mitochondria in Bcl-x_L cells by this time point and almost total release 12 h post-treatment. The data are summarized in Figure 2b. It may be noted that the Neo- and Bcl-x_L-expressing lines used in our experiments were

clonal isolates. Additionally, the Bcl-x_L lines were selected for high levels of expression of the transfected plasmid.[22] Under conditions of IL-3 withdrawal, these cell lines show mitochondrial retention of cyt *c* for at least 72 h postdeprivation (not shown). Since FL5.12 control cells primarily adopt the (type II) cyt *c*-dependent caspase-9 pathway in the amplification of downstream apoptotic cascades in response to TNF-α, it was important to determine the mechanism underlying the early and rapid apoptosis of the Bcl-x_L-expressing cells in the absence of cyt *c* release.

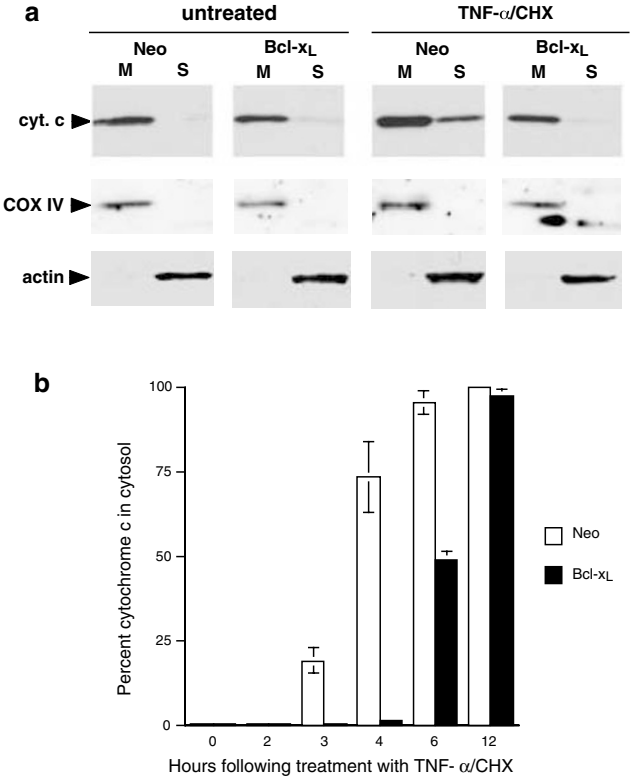


Figure 2 Bcl-x_L prevents early mitochondrial cytochrome *c* release in FL5.12 cells in response to TNF-α/CHX treatment. (**a**) Western blot of fractionated FL5.12 cells showing the distribution of cyt *c*. Untreated Neo and Bcl-x_L FL5.12 cells, and cells treated with TNF-α/CHX for 3 h were fractionated into mitochondrial (M) and cytoplasmic, S100 (S), fractions, and immunoblotted with a monoclonal antibody against cyt *c*, COX IV and actin (see Materials and Methods). (**b**) Bar graph showing the ratio of cytosolic cyt *c* to total cyt *c* in Neo and Bcl-x_L FL5.12 cells 0, 2, 3, 4, 6 and 12 h following apoptotic induction with TNF-α/CHX (mean and standard deviations, n=3). Fractionated cell extracts were immunoblotted with antibody against cyt *c* and the chemiluminescent cyt *c* bands, visualized by autoradiography, were later quantified by densitometry using a BioRadGS-363 Molecular Imager

Caspase-9 is processed prior to mitochondrial cyt *c* release in response to TNF-receptor occupation in Bcl-x_L-expressing FL5.12 cells

Next, we examined the processing of caspase-9 and -3, the two major proteases associated with mitochondrial and nonmitochondrial pathways downstream of activated caspase-8, under conditions of TNF-receptor crosslinking. Figure 3 shows lysates of FL5.12 control and Bcl-x_L cells at 0, 2, 3, 4 and 6 h after treatment with TNF-α/CHX, immunoblotted with antibodies against caspase-9 (upper panels) or a cleaved (active) form of caspase-3 (lower panels). Caspase-9 is being processed within the first 2 h of induction in both the cell lines. We have consistently observed the early cleavage of caspase-9 in response to TNF-α; the results shown in Figure 3 are representative of six different experiments. A number of groups have already established that caspase-9 requires cyt *c* release and recruitment into the apoptosome to autoprocess and become activated.[12,23] However, data in Figure 2 showing effective inhibition of cyt *c* release by overexpressed Bcl-x_L for three or more hours after induction of apoptosis through the TNF receptor (Figure 2), taken together with results in Figure 3, strongly indicate that caspase-9 is processed prior to mitochondrial cyt *c* release in the FL5.12 cells. The cleaved caspase-3 (Figure 3, lower panels) detected within the first 3 h after induction probably results from caspase-8 processing activity. It may be noted that levels of this cleaved, presumably active, form increase noticeably at later time points in both vector and Bcl-x_L-expressing cells, and this was confirmed using a colorimetric caspase-3 activity detection kit (R&D Systems, not shown). Additionally, it may be noted that the 38 kDa processed product of caspase-9 consistently appeared at least as early after TNF treatment as the 40 kDa cleavage product expected from caspase-3 processing activity (Figure 3, upper panels). It was important to determine whether the observed early processing of caspase-9 was leading to its activation or was merely a consequence of the degradative activity associated with apoptotic progression.

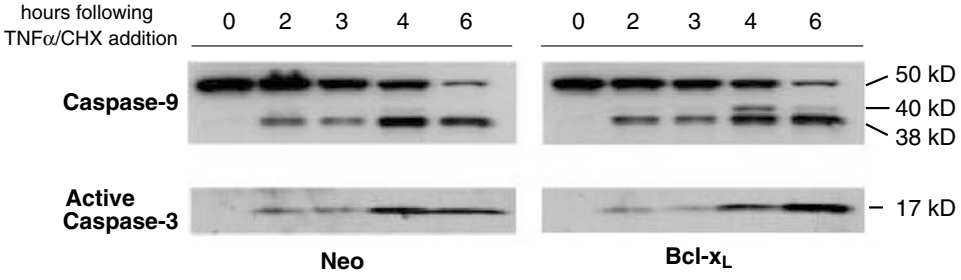


Figure 3 Caspase-9 processing in FL5.12 Bcl-x_L cells occurs within 2 h of apoptotic induction with TNF-α/CHX. Autoradiographs of lysates from Neo and Bcl-x_L FL5.12 cells treated with TNF-α/CHX for 0, 2, 3, 4, 6 and 12 h immunoblotted with antibody against caspase-9 (top panels) or a cleaved form of caspase-3 (bottom panels)

Early caspase-9 activity contributes to the rapid amplification of apoptosis in Bcl-x_L-expressing FL5.12 cells in response to TNF-α

We predicted that if early caspase-9 activation was critical for the rapid and early amplification of this apoptotic pathway, inhibiting caspase-9 activity would delay apoptosis. To test this, FL5.12 cells were treated with TNF-α and CHX in the presence of either the pan-caspase inhibitor z-VAD-fmk, or specific caspase inhibitors. Shown in Figure 4a are bar graphs depicting the viability of FL5.12 cells at 6 h following TNF-α treatment, as assayed by flow cytometric analysis of Annexin V and PI uptake. As expected, z-VAD-fmk blocked apoptosis of the Bcl-x_L cells while providing only partial protection to the controls. It has previously been shown that 50 μM z-VAD-fmk is unable to block cyt *c* release in FL5.12 Neo cells[15] (and AK, unpublished). The caspase-3-specific inhibitor, z-DQMD-fmk, imparted 30–50% protection over the TNF-α/CHX controls in both the cell lines. It is known that related effector caspases, such as caspase-7, can effectively replace active caspase-3[24,25] in processing downstream targets, and this may be the reason for the lower levels of protection observed in the presence of z-DQMD-fmk. The caspase-8 inhibitor, z-IETD-fmk, was more effective in inhibiting apoptosis in both the cell lines. Figure 4a also shows that z-LEHD-fmk, an inhibitor of caspase-9 activity, delayed apoptosis almost as efficiently in the Bcl-x_L cells as did z-IETD (right panel), suggesting that caspase-9 proteolytic activity plays an important role in this death pathway even in the absence of cyt *c* release. The low level of inhibition observed in control FL5.12 cells (Figure 4a, left panel) in the presence of the caspase-9 inhibitor can be attributed to the other proapoptotic compounds, such as Smac/DIABLO, AIF, and even preprocessed caspase-3, that are released from the mitochondrial intermembrane space along with cyt *c*. Although, IETD and LEHD were able to delay the onset of apoptosis more effectively than the caspase-3 inhibitor, only the broad-spectrum inhibitor z-VAD could protect the Bcl-x_L cells against TNF-induced death for over 24 h (not shown). Figure 4b is a Western blot of FL5.12 Bcl-x_L cell lysates showing caspase-9 and -3 processing, 3 and 6 h following TNF-α/CHX treatment in the presence or absence of caspase inhibitors. While the caspase-8 inhibitor, IETD, effectively prevented processing of both caspase-9 and -3 (lanes 6 and 7), the caspase-3 inhibitor (lanes 4 and 5) did not affect the processing of either of these caspases. Figure 4c (top panel) represents a caspase-9 immunoblot of lyates from untreated FL5.12 Bcl-x_L cells, and cells treated with TNF-α/CHX in the presence of 50 μM z-VAD. The membrane was sequentially stripped and reprobed with antibodies against active caspase-3 and actin (center and bottom panels, respectively). As expected, based on the viability assays shown in Figure 4a (right panel), both caspase-9 and -3 processing were completely inhibited.

Although caspase-9 inhibition effectively blocked the processing of caspase-3 at early times, by the end of 6 h levels of cleaved caspase-3 appeared to have been restored to those observed in the controls (Figure 4b, lower panel, lanes 8 and 9). To resolve this issue, DEVDase activity was measured in TNF-treated FL5.12 Bcl-x_L cells in the presence

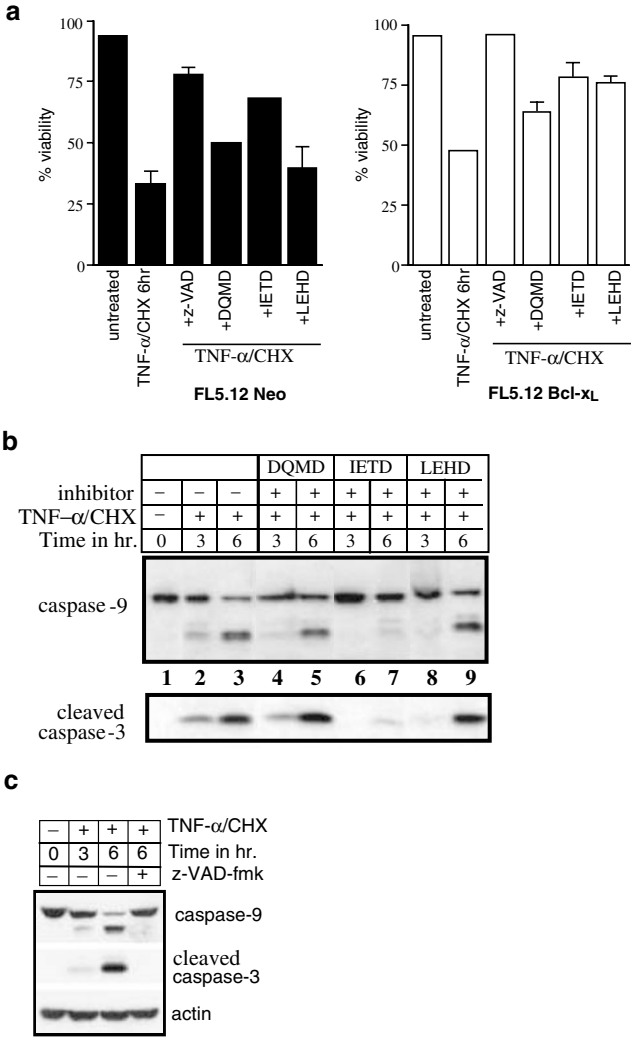
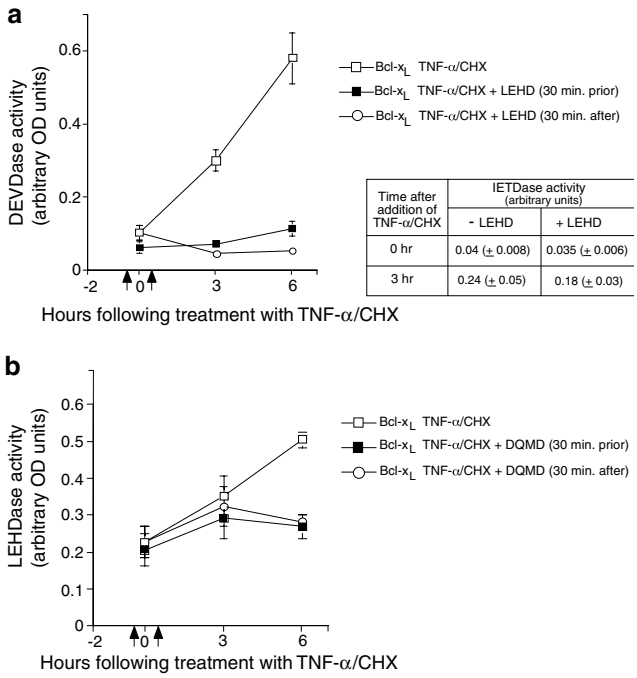


Figure 4 Early caspase-9 processing contributes to the rapid amplification of apoptosis observed in Bcl-x_L-expressing FL5.12 cells in response to TNF-α/CHX. (**a**) FL5.12 cells were incubated with 100 μM concentrations of specific cell-permeable caspase inhibitors z-DQMD-fmk (caspase-3), z-IETD-fmk (caspase-8) or z-LEHD-fmk (caspase-9) and 50 μM of the pancaspase inhibitor z-VAD-fmk for 30 min before being treated with TNF-α and CHX. Viability of cells was determined at specific intervals by flow cytometric analysis Annexin V–FITC labeling and PI uptake (see Materials and Methods). Figure represents percent viability of untreated FL5.12 Neo (left panel) and Bcl-x_L (right panel) cells, and cells treated with TNF-α/CHX for 6 h in the absence or presence of caspase inhibitors (mean and standard deviations, n=3). (**b**) Western blots of lysates from 0, 3 and 6 h-treated FL5.12 Bcl-x_L cells from the experiment in (4**a**). Top panel shows an autoradiograph of 25 μg total protein immunoblotted with antibody against caspase-9, and bottom panel represents the same Western blot, stripped and reprobed with an antibody against the cleaved (active) form of caspase-3. (**c**) Caspase-9 immunoblot of z-VAD-fmk-incubated Bcl-x_L cell lysates (top panel) from the above experiment, stripped and reprobed with active caspase-3 (center panel) and actin (bottom panel) antibodies

of LEHD added 30 min before or after the addition of inducer (Figure 5a). Results indicated that caspase-3 activity was effectively blocked at the earliest time point in both the instances. Activity remained low in both samples at the end of 6 h, and consistently showed steady increases in DEVDase levels thereafter (not shown). If caspase-3 was being directly inhibited by LEHD rather than through inhibition of caspase-9, we should also detect caspase-8 inhibition, since caspase-8



Time after addition of TNF-α/CHX	IETDase activity (arbitrary units)	
	- LEHD	+ LEHD
0 hr	0.04 (± 0.008)	0.035 (± 0.006)
3 hr	0.24 (± 0.05)	0.18 (± 0.03)

Figure 5 Inhibition of caspase-9 inhibits early DEVDase activity, but inhibition of caspase-3 does not affect early LEHDase activity in FL5.12 Bcl-x$_L$ cells treated with TNF-α/CHX. Cells were incubated with 100 μM z-LEHD-fmk (**a**) or z-DQMD-fmk (**b**) 30 min prior to, or 30 min. following (shown by arrows) the addition of TNF-α/CHX, and DEVDase (**a**) or LEHDase (**b**) activity measured at the indicated time points using colorimetric assays (R&D Systems). Table inset in (**a**) shows IETDase activity measured at 0 and 3 h in the FL5.12 cells treated with z-LEHD-fmk prior to apoptosis induction

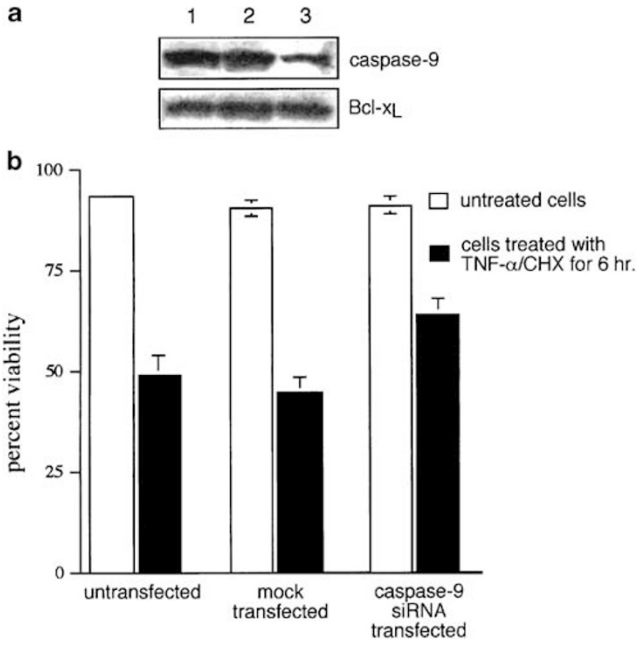


Figure 6 Downregulation of caspase-9 levels by RNA interference partially protects FL5.12 Bcl-x$_L$ cells against TNF-receptor-activated apoptosis. FL5.12 Bcl-x$_L$ cells were either mock transfected or transfected with mC-9 siRNA (100 nM) using siPORT Amine (Ambion) as transfection agent. (**a**) Western blot of lysates from parental FL5.12 Bcl-x$_L$ cells (lane 1), mock-transfected FL5.12 Bcl-x$_L$ cells (lane 2) and cells transfected with caspase-9 siRNA (lane 3). Upper panel represents an immunoblot of caspase-9 and lower panel shows the same blot stripped and reprobed with an antibody against the Bcl-x$_L$ protein. (**b**) Untransfected, mock-transfected and caspase-9 siRNA-transfected FL5.12 Bcl-x$_L$ cells were treated with TNF-α and CHX and viability of cells was determined by flow cytometric analysis of Annexin V–FITC labeling and PI uptake. Bar graphs represent percent viability of unstimulated and 6 h-stimulated cell populations (n=3)

and -9 are more closely related in terms of their substrate specificities.[26] However, IETDase activity measured at 0 and 3 h in the FL5.12 cells treated with LEHD prior to apoptosis induction, indicated that caspase-8 was not significantly inhibited at the 3 h time point (Table inset, Figure 5a). The 25% reduction observed in IETDase activity is probably responsible for part of the decrease in caspase-3 activity, but we believe that these data indicate that a bulk of the *early* inhibition of caspase-3 activity results from inhibition of caspase-9 function.

Western blots of caspase-9 in Figure 4b (upper panel, lanes 4 and 5) showed no effect of DQMD, the caspase-3-specific synthetic inhibitor, on caspase-9 processing. Colorimetric caspase-9 activity assays in the presence and absence of DQMD (Figure 5b) showed that caspase-3 inhibition did not block early caspase-9 activation, but inhibited late LEHDase activity. These results confirm that caspase-9 is activated within 3 h following TNF-receptor crosslinking and suggest, furthermore, that active caspase-3 is not the protease responsible for the early activation.

RNA interference-mediated downregulation of endogenous caspase-9 protects FL5.12. Bcl-x$_L$ cells from TNF-receptor-activated death

Although data in Figure 4 were suggestive of caspase-9 participation in early amplification of apoptosis in FL5.12 Bcl-x$_L$ cells, it was important to confirm this by methods that did not involve the use of synthetic caspase inhibitors. For an alternative approach, FL5.12 Bcl-x$_L$ cells were transiently transfected with siRNA directed against nucleotides 289–309 of the murine caspase-9 (mC-9) RNA sequence (see Materials and Methods). Figure 6a shows a caspase-9 immunoblot of lysates 48 h following transfection from parental, mock transfected and siRNA-transfected FL5.12 Bcl-x$_L$ cells (upper panel). The membrane was stripped and reprobed with an antibody against Bcl-x$_L$ to confirm even loading of lysates (lower panel). The decrease in caspase-9 protein expression was determined by densitometry to be roughly 50%. Figure 6b shows the results of viability assays carried out on control and transfected populations treated with TNF-α and CHX for 6 h. While the mean viability exhibited by the mock-transfected cells did not differ from the 48% viability observed in the untransfected parental population, caspase-9 siRNA-transfected cells were 64% viable at the end of 6 h. The observed delay in apoptosis in the presence of reduced levels of caspase-9 supports earlier results (see Figures 4 and 5) that caspase-9 function is involved in accelerating TNF-activated apoptosis in cells in which the mitochondrial cyt *c* release pathway is effectively blocked.

Early caspase-9 processing occurs in cells lacking the Apaf-1 protein

Having determined that the early proteolytic activity of caspase-9 was playing a role in the amplification of apoptosis in the Bcl-x_L-transfected cells, we proceeded to determine the source of this early activation. It was unlikely that active caspase-3 molecules resulting from the proteolytic activity of caspase-8 were acting on procaspase-9 to process and activate it (Figures 4 and 5). It was possible that a constitutively active form of Apaf-1 was being generated in response to receptor occupation that could bypass the requirement for cyt *c* association in order to activate the autoprocessing of procaspase-9 molecules. We surmised that if Apaf-1 was required for the early activation of caspase-9 in the TNF-α pathway, then early processing of caspase-9 should not be detectable in Apaf-1-deficient cells exposed to TNF. Additionally, by using Apaf-$1^{-/-}$ cells, we would ensure that no activation of caspase-9 could occur via the cyt *c* release pathway, while eliminating the need for overexpressing Bcl-x_L to block this pathway. Figure 7 shows Western blots of lysates from Apaf$^{-/-}$ and control mouse embryo fibroblasts (MEFs) that had been treated with TNF-α/CHX in the presence or absence of z-VAD-fmk, the broad-spectrum caspase inhibitor. Upper panel in Figure 7 shows that caspase-9 is processed in both types of MEFs within 3 h following exposure to TNF-α and CHX. Although, z-VAD completely inhibits this processing in Apaf-$1^{-/-}$ cells, control MEFs continue to activate caspase-9 at least partially via the mitochondrial pathway. The Western blot was stripped and reprobed with an antibody against Apaf-1 (Figure 7, lower panel). These data suggest that the early cyt *c*-independent processing of caspase-9 in the TNF pathway in these cells did not result from Apaf-1 activity.

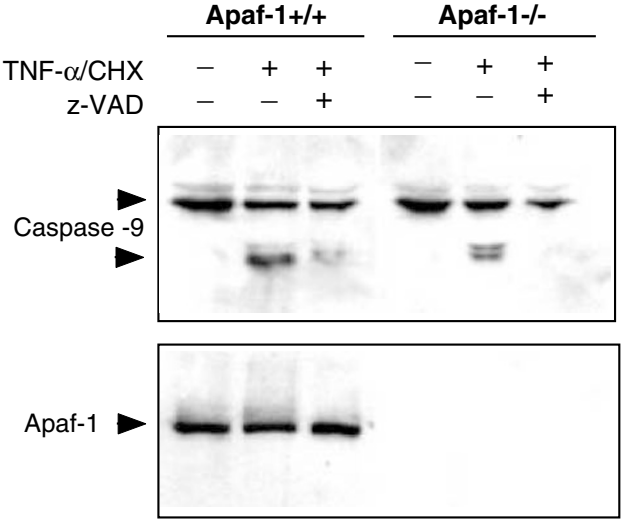


Figure 7 Early caspase-9 processing in murine fibroblasts following exposure to TNF-α/CHX is dependent on caspase activity and not on the Apaf-1 protein. The figure shows autoradiographs of Western blots from lysates of Apaf$^{+/+}$ and Apaf$^{-/-}$ MEFs that had been treated with TNF-α/CHX for 3 h in the presence or absence of the caspase inhibitor z-VAD-fmk. Upper panel shows caspase-9 processing, and lower panel shows the same Western blot, stripped and reprobed with an antibody against Apaf-1 (see Materials and Methods)

Murine caspase-9 is processed by active caspase-8 in an *in vitro* cleavage assay

Yet another possibility was that active caspase-8 molecules were themselves directly acting on procaspase-9 to process and activate it. Although caspase-8 had been shown to activate caspase-9 *indirectly* in type II cells via cleavage of the Bid protein and the subsequent release of mitochondrial cyt *c*, to date there has been no evidence of *direct* processing of caspase-9 *in vivo* by this initiator caspase.

The autoprocessed form of caspase-9 is 38 kDa (see Figure 3, upper panels), but autoprocessing of the latter has only been shown to occur *in vivo* by 'induced proximity' in the context of apoptosome formation in reponse to cyt *c* release.[23,27] The autoprocessing motif, SEPD (residues 350–353), in mC-9 is preceded directly by LDSD (346–349), both putative caspase-8 cleavage consensi[26] (see Figure 8a). The LDSD motif is absent in the human caspase-9 (hC-9) protein.

Full-length murine procaspase-9 was cloned from mouse brain cDNA and tagged with a myc epitope at the C-terminus. Two point mutants of murine caspase-9, LDSA and SEPA, were also generated in which residues D^{349} and D^{353}, respectively, were replaced with alanine. Radiolabeled *in vitro* translated murine caspase-9, LDSA and SEPA, as well as *in vitro* translated human wild-type protein were incubated either with active recombinant caspase-3 or -8 at 37°C for 1 h. Figure 8b shows an autoradiograph of SDS-PAGE analysis of the reactions. Active caspase-3 (lanes 5–8) processed both the human and murine proteins generating cleavage products of the expected size; 40 kDa for murine caspase-9 and 37 kDa for the human protein. The panel on the right (lanes 9–12) shows the results of active caspase-8 processing activity on caspase-9 and the two point mutants. A major processed fragment of 38 kDa and a minor 40 kDa product resulted from cleavage of wild-type mC-9 as well as the LDSA mutant. The 38 kDa fragment generated by caspase-8 cleavage activity is absent in the cleavage reaction with the SEPA mutant (lane 11) indicating that it was the SEPD, and not the LDSD, motif that served as a cleavage site for active caspase-8. Two cleavage products of approximately 37 and 35 kDa, resulting from processing at the DQLD and PEPD motifs, were also observed in the cleavage reaction with human caspase-9 (lane 12), however, processing at PEPD by active caspase-8 was consistently weaker than at the corresponding (SEPD) motif in the murine protein (lane 9). Srinivasula *et al*.[28] have demonstrated the cleavage of purified recombinant hC-9 by active caspase-8, and their data indicate that although hC-9 is processed at both sites, the DQLD motif is the preferred site of cleavage. Thus, mC-9 serves as a direct processing substrate for active caspase-8, and may be one of the first caspases to be activated in the TNF pathway of apoptosis.

Discussion

Human cells have been classified as cells of type I or type II based on the nature of their response to death receptor activation by ligands such as Fas and TNF-α.[5] In cells of type I death receptor ligation leads to vigorous activation of caspase-8, followed by the direct cleavage and activation of

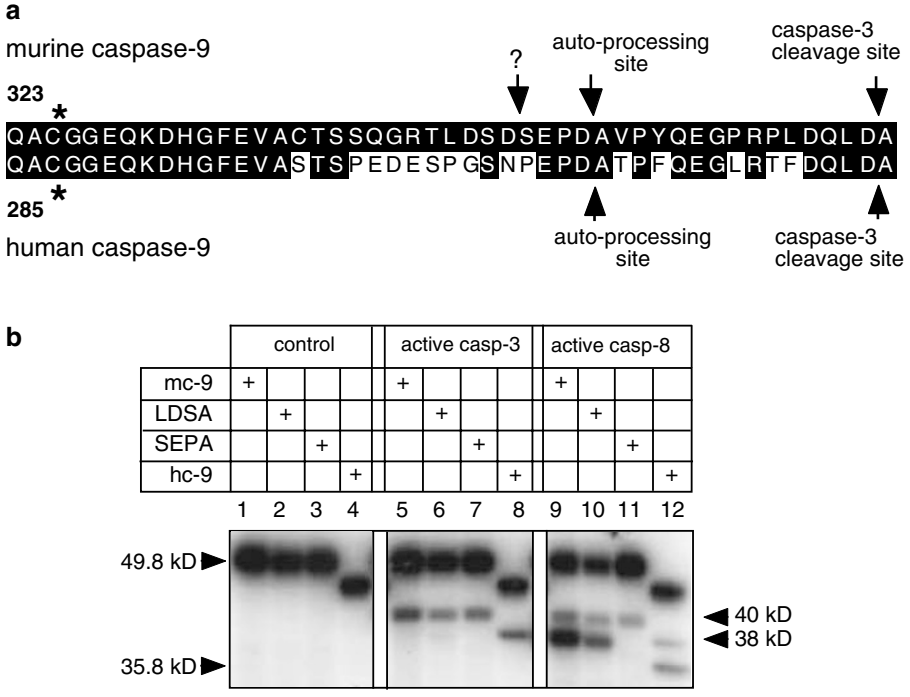


Figure 8 Active recombinant caspase-8 cleaves murine caspase-9 at SEPD, the site of caspase-9 autoprocessing. (**a**) Representation of the amino-acid sequence between the active site, QACGG (*), and the caspase-3 processing site (marked by arrows) in mC-9 and hC-9. Conserved residues are shaded in black. Additional arrows depict the caspase-9 autoprocessing sites, SEPD (mouse), PEPD (human) and a putative caspase-8 cleavage consensus motif, LDSD (?) present only in the murine protein. (**b**) *In vitro* synthesized, radiolabeled mC-9, LDSA, SEPA or hC-9 proteins were synthesized using the TNT transcription/translation system (Promega) and incubated with 15 mU/μl of active human recombinant caspase-3 (lanes 5–8), or 150 mU/μl of active caspase-8 (lanes 9–12) at 37°C for 60 min. Lanes 1–4 show the 'input' or corresponding labeled translation products that went into each reaction. Cleavage reactions were resolved by SDS-PAGE, and the gels fixed, dried and autoradiographed

procaspase-3.[5,7] DISC assembly in type II cells, on the other hand, is both delayed and subdued and the resulting autoprocessed caspase-8 insufficient for the direct activation of caspase-3. Caspase-8 can still cleave the Bcl-2 family protein, Bid, however, to generate a truncated active form of the latter that translocates to the mitochondria and promotes the release of mitochondrial cyt *c* into the cytosol.[8-10] Most activated effector caspases have the ability to cleave other caspases, including initiator caspases, but such processing activity is unregulated and primarily associated with the degradative phase of the apoptotic process. Caspase-2, -6 and -9, for instance, are efficiently processed by activated caspase-3 during the rapid cell death observed in type I cells in response to Fas ligand.[24,29] In these cells, caspase-9 functions not as an initiator, but as one more target of an effector caspase. Caspase-9 activation follows that of caspase-3 in such a pathway. Survival members of the Bcl-2 family, by virtue of their ability to prevent cyt *c* release, therefore, impart almost total protection against apoptosis induced via Fas receptor crosslinking to type II cells. The murine FL5.12 cell line in the present study exhibits both type I and type II characteristics. Preventing cyt *c* release by overexpressing an antiapoptotic Bcl-2 family protein, does not protect FL5.12 cells from death receptor activation-induced apoptosis[15] (and Figure 1). Given the close conservation of specific death pathways among humans and mice, as well as the high degree of homology shared by components of different apoptotic pathways among the two species, it was important to investigate further the biochemical basis for this difference in responsiveness.

Apoptosis is discernible by flow cytometry within 2 h of addition of TNF-α/CHX, even as overexpressed Bcl-x_L is able to prevent mitochondrial cyt *c* release for at least 4 h in FL5.12 cells. However, while Johnson *et al*.[15] observed no cyt *c* release for 6 h in response to TNF-α/CHX, our studies show that Bcl-x_L FL5.12 cells release almost 50% of their cyt *c* into the cytoplasm by the end of 6 h under the same conditions (Figure 2b). The FL5.12 Bcl-x_L lines used in the present study have been selected for high-level expression of transfected Bcl-x_L[22] and have consistently exhibited this delayed release of cyt *c* in response to TNF-α, while protecting mitochondria for over 72 h following growth factor (IL-3) withdrawal. We believe that the positive feedback of the cytosolic caspase activity on the mitochondria causes the release of cyt *c* as a late event during the apoptotic process.[30] This feedback loop could be manifest in a number of ways, including cleavage of the membrane-inserted survival-promoting proteins themselves.[31,32]

We show that caspase-9 is processed within 2 h of activation of the death receptor pathway in Bcl-x_L-expressing FL5.12 cells. Additionally, using specific inhibitors we observe that caspase-9 activity is essential for the early amplification of apoptosis, under these circumstances. This processing of caspase-9 proceeds in the absence of mitochondrial cyt *c*

release and occurs too early to be the result of a feedback loop involving active caspase-3.[24] Furthermore, the Western blots in Figure 3 (lower panels) show relatively low amounts of cleaved/active caspase-3 resulting from caspase-8 processing activity at 2 and 3 h as compared to the cleaved form detected at later time points. Additionally, the first detectable processing intermediate is the 38 kDa product, and not the 40 kDa band expected from caspase-3 cleavage activity. The processing of procaspase-9 by active caspase-3 (at residue D^{368}), known to occur during amplification of caspase cascades, generates a 40 kDa cleavage product in the *in vitro* cleavage assays (shown in Figure 8b), and confirms this. The inhibitor studies further confirm that active caspase-3 is not the protease responsible for the observed early caspase-9 processing (Figures 4b and 5b). Procaspase-9 has been shown to be cleaved as cells undergo apoptosis; however, such cleavage occurs late during apoptotic progression and is thought to be involved in the turnover of the protease rather than in its enzymatic activation.[17,33]

Apaf-1 oligomerization in the presence of cyt *c* and dATP has been shown to be the primary cause of the autoprocessing that results in the activation of caspase-9.[12,23,34]. Although the full-length Apaf-1 molecule requires cyt *c* for oligomerization, a truncated form of Apaf-1 lacking the WD-40 repeat region has been shown to be constitutively active *in vitro*.[18] We were able to detect truncated forms of Apaf-1 *in vivo* in response to TNF-α/CHX in the FL5.12 cells (not shown), but these truncated forms appeared 3–4 h after the initiation of the signal, too late to initiate caspase-9 autoprocessing and were more likely the result of caspase-3 processing activity.[35,36] Furthermore, we could demonstrate that caspase-9 cleavage occurs within 3 h of TNF-receptor occupation in Apaf-1-deficient MEFs (Figure 7).

Caspase activity determinations in both FL5.12 Bcl-x_L cells and Apaf-$1^{-/-}$ MEFs, using colorimetric substrates, demonstrated that the processed caspase-9 protein had caspase activity (Figure 5b, and not shown). Additionally, FL5.12 Bcl-x_L cells expressing half as much caspase-9 protein as the parental controls, showed 30% increased viability when exposed to TNF-α/CHX for 6 h (Figure 6). The partial protection imparted by downregulated caspase-9 is strong indication that the activity of this caspase contributes to the early, rapid apoptosis observed in the parental cells (Figure 1b).

The 38 kDa processing intermediate of caspase-9 appears to be a product of caspase-8 cleavage activity. *In vitro* cleavage assays, using active recombinant caspase-8, confirm that the 38 kDa band is a major processing product and is a result of cleavage at the SEPD (D^{353}) and not the LDSD motif (D^{349}) on murine caspase-9 (Figure 6). Furthermore, the early appearance of the shorter processing intermediate, in TNF-treated FL5.12 cells, supports the possibility that caspase-9 is a direct processing substrate for active caspase-8 in this pathway of apoptosis. *In vitro* cleavage data demonstrate that human caspase-9 is also cleaved, albeit less efficiently than its murine counterpart, suggesting that the SEPD motif serves as a better substrate for active caspase-8 than does the PEPD site (Figure 6a). Previously published studies demonstrating the processing of purified recombinant hC-9 by active caspase-828, indicate that DQLD (D^{330}), rather than PEPD (D^{315}), is the preferred site of cleavage activity. This difference in susceptibility between hC-9 and mC-9 to processing by active caspase-8 may help explain the difference in response to death receptor activation between human cells of type I and murine FL5.12 cells. Additional evidence that hC-9 and mC-9 are differently regulated comes from a study showing that the Akt phosphorylation site (located at $Serine^{196}$) presumed to be involved in the inactivation of hC-9 is absent from its murine counterpart.[37,38]

In mitochondrial pathways of apoptosis, the zymogen form of caspase-9 is rapidly recruited into the apoptosome following the release of cyt *c*.[39] The active form of caspase-9 is believed to be the Apaf-1-bound holoenzyme. Since procaspase-9 is not normally detected in the apoptosome, it is thought to undergo rapid autoprocessing. However, studies with noncleavable mutants have shown that the unprocessed caspase can also associate with oligomerized Apaf-1 and rapidly recruit and activate caspase-3.[16,17] It is unlikely that a holoenzyme is involved in the processing and activation of caspase-9 in response to TNF-α in the FL5.12 cells. Our data suggest, instead, that caspase-9 is functioning not as an initiator, but as an effector caspase and as a direct substrate for active caspase-8 early after apoptotic stimulation. The possibility that the murine caspase-9 cleavage site is modified in healthy, proliferating cells in a manner similar to the Bid protein is an attractive one and currently under investigation.

We propose the following model to explain our observations. In addition to cleaving the Bcl-2 protein Bid and small amounts of procaspase-3, active caspase-8 molecules in FL5.12 cells cleave and activate procaspase-9 in response to TNF-receptor crosslinking (see Figure 9). The cleaved Bid (tBid) promotes the release of cyt *c*, causing rapid amplification of apoptosis via apoptosome formation and caspase-9 activation.[8-10] In the absence of cyt *c* release, however, caspase-9 that has been processed directly by caspase-8 cleaves and activates caspase-3, which, in turn, processes more caspase-9 in a positive feedback amplification loop. It may be argued that a cell overexpressing Bcl-x_L is not a 'normal' cell and cannot, therefore, serve as a model for understanding mechanisms of apoptosis. We believe we have identified a novel pathway of amplification of the caspase

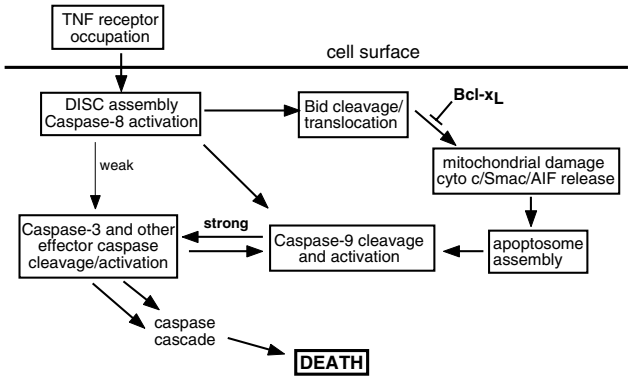


Figure 9 Simplified model of the apoptotic pathways activated following TNF receptor occupation in murine cells

cascade; in order to appreciate the cyt *c* -independent nature of the process, it is important to use a cell line in which cyt *c* release can be prevented or delayed. Although we have investigated this pathway in a murine pro-B system, similar mechanisms are undoubtedly utilized in other murine cell types. Apaf-1-deficient fibroblasts offer insight into alternate pathways since cyt *c*-induced activation is also effectively shut off in these cells. It is also relevant to note that a number of cancers resistant to apoptotic induction overexpress anti-apoptotic Bcl-2 proteins,[40] and activating the apoptotic process in such cells by alternative means can be an important goal in cancer therapy.

A recent study showed that caspase-12 was able to process and activate caspase-9 in response to ER stress,[20] while another indicated that Sendai virus infection could trigger a novel, yet unidentified, pathway of caspase-9 cleavage.[19] We have presented evidence that implicates caspase-8 in the processing and activation of caspase-9 in death receptor-activated pathways. All three studies clearly indicate that caspase-9 can also be activated by mechanisms involving neither cyt *c* release nor Apaf-1 activation. In this capacity, the activated protease probably contributes to the amplification of death pathways that have already been initiated. A cell programmed to die utilizes all the means within its control to die a quick death. It stands to reason, therefore, that proteins, such as caspase-9, functioning at critical points of apoptotic pathways have the ability to amplify the apoptotic process by more than just one mechanism to ensure the cell's early and unobtrusive demise.

Materials and Methods

Cell lines, antibodies and plasmid constructs

FL5.12 murine pro-B lymphoma cells were cultured as described earlier.[41] For plasmid transfections, 1×10^7 FL5.12 cells were electroporated at 960 μF and 250 V with 10 μg of plasmids, pSFFV-Neo or pSFFV-Bcl-x_L, described previously.[42] Neomycin-resistant cells were selected in medium containing 1 mg/ml G418. Single cell clones were obtained from transfectant pools by limiting dilution cloning in 96-well microtiter plates. Apaf-1 knockout and control primary embryo fibroblasts (a gift from Scott Lowe, Cold Spring Harbor) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM β-mercaptoethanol. Active recombinant caspase-3 was from Pharmingen and active caspase-8 from BioVision, Inc.

Caspase-9 monoclonal antibody, cleaved caspase-3 polyclonal antibody and the Apaf-1 monoclonal antibody were purchased from Stressgen Biotechnologies, Cell Signaling Technology, and Chemicon International, respectively. The cyt *c* antibody was a gift from R Jemmerson (University of Minnesota, USA). The COX IV antibody was purchased from Molecular Probes and the actin antibody was obtained from Oncogene. The hC-9 construct was a gift from E Alnemri (Thomas Jefferson University). Full-length mC-9 cDNA was synthesized from mouse brain RNA by PCR using specific oligos, and cloned into the *Bam*HI and *Xho*I sites of the pCDNA 3.0 vector (Invitrogen). The myc-tagged construct, mC-9myc, was generated by subcloning mC-9 into *Bam*HI and *Xho*I sites of a pCmyc vector (gift from G Nunez, University of Michigan, USA). The two point mutants of mC-9myc (LDSA and SEPA) converting D^{349} or D^{353}, respectively, to alanine, were generated by a two-step PCR method.

Cyt *c* release studies

Mitochondrial and cytosolic (S100) fractions were prepared by resuspending 1×10^7 FL5.12 cells in 0.8 ml ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml aprotinin, 2 μg/ml leupeptin (pH 7.4)). Cells were homogenized using a prechilled cylinder cell homogenizer (H&Y Enterprise Redwood City, CA, USA), following which the unlysed cells and nuclei were pelleted at 750 $\times$ g for 25 min. The pellet, representing the mitochondrial fraction was resuspended in buffer A, and the supernatant subjected to further centrifugation at 100 000 $\times$ *g* for 1 h. The supernatant from the final centrifugation represented the cytosolic fraction. Equivalent amounts of mitochondrial and cytosolic (S100) fractions were then Western blotted as previously described, first with an antibody against cyt *c* (gift from R Jemmerson) followed by an antibody against COX IV (Molecular Probes).

Cell viability and caspase activity assays

Viability of FL5.12 cells was measured at 2 or 3 h intervals over a period 24 h following the addition of TNF-α (5 ng/ml) and CHX (20 μg/ml) to the medium, or at 24 h intervals for 4 or 5 days following withdrawal of IL-3 from the medium.[41] For the caspase inhibitor studies, FL5.12 cells were incubated with z-DQMD-fmk, z-IETD-fmk or z-LEHD-fmk at 100 μM concentration or z-VAD-fmk (50 μM) for 30 min prior to the addition of TNF-α and CHX. For the IL-3 studies, cells were washed three times in growth medium lacking IL-3 and resuspended in this medium at a concentration of 5×10^5 cells/ml. Aliquots were withdrawn at specified time intervals, and viability determined by flow cytometry using PI exclusion alone or a combination of Annexin V–FITC uptake and PI exclusion using protocol suggested by BioVision, Inc. DEVDase, IETDase and LEHDase activities in cell lysates (from 2×10^6 cells per data point) were determined using colorimetric assay kits (R&D Systems) according to the manufacturer's protocol.

Western blotting

FL5.12 cells were pelleted, washed in phosphate-buffered saline, and then lysed in RIPA buffer containing 1% Nonidet P-40, 1% deoxycholate and 0.1% SDS, supplemented with protease inhibitor cocktail (Calbiochem). Adherent MEFs were detached from culture plates with Accutase (Innovative Cell Technologies, Inc.) and pelleted, together with predetached floaters, as described above. The lysed cells were centrifuged at 14 000 $\times$ *g* to remove cellular debris. Protein concentrations of extracts were determined by the colorimetric bicinchoninic acid method (Pierce Chemical Company). Equal amounts of protein were electrophoretically separated in 14% SDS-PAGE and transferred to nitrocellulose. Membrane blocking, washing, primary and secondary antibody incubations and chemiluminescence reactions were carried out according to the Amersham ECL protocol. Antibody dilutions were carried out as suggested on the data sheet provided by the manufacturing company. Blots were stripped for reuse by washing for 30 min to 2 h in TBS-T buffer (pH 3.0) at room temperature.

siRNA construction and transfection

siRNAs were generated according to the protocol accompanying the Silencer siRNA kit (Ambion, Inc.). Briefly, sense and antisense oligos were synthesized to target a 21-nucleotide mC-9 sequence (AAGCAGGATC-CAGAGGCTGTT-3′), with an additional 8-nucleotide leader sequence complementary to a T7 promoter primer. Each oligo was hybridized to the

T7 promoter primer, filled in with Klenow, and used as a template for transcription by T7 polymerase. Sense and antisense *in vitro* transcripts were hybridized, following which overhanging leader sequences and residual DNA templates were removed by treatment with RNase and Dnase, respectively. Finally, the double-stranded RNAs were purified by passing through a filter cartridge and eluted in nuclease-free water. FL5.12 cells were either mock-transfected or transfected with the siRNA (100 nM) in the presence of transfection agent siPORT *Amine* (Ambion, Inc.) for 6 h. A fraction of the transfected population was lysed and immunoblotted with caspase-9 antibodies, and the remaining cells were used for the viability studies described above.

In vitro caspase cleavage assays

Radiolabeled mC-9-myc protein, LDSA, or SEPA proteins were synthesized *in vitro* using the TNT transcription/translation system (Promega) and 20 μCi [^{35}S]methionine (Amersham/Pharmacia) per reaction. Active human recombinant caspase-3 (15 mU/μl), or -8 (150 mU/μl), was used to cleave the *in vitro* translated proteins at 37°C for 60 min using 5 μl *in vitro* translation mix as substrate in a 25 μl total reaction volume. Reactions were stopped with an equal volume of 2 × Laemmli sample buffer containing reducing agent. The resulting cleavage products were separated by SDS-PAGE, and the gels were fixed, dried and autoradiographed.

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