Apoptosis 2003; **8**: 363–370

Caspase-3 activation is an early event and initiates apoptotic damage in a human leukemia cell line

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Many apoptotic pathways culminate in the activation of caspase cascades usually triggered by the apical caspases-8 or -9. We describe a paradigm where apoptosis is initiated by the effector caspase-3. Diethylmaleate (DEM)-induced apoptotic damage in Jurkat cells was blocked by the anti-apoptotic protein Bcl-2, whereas, a peptide inhibitor of caspase-3 but not caspase-9 blocked DEM-induced mitochondrial damage. Isogenic Jurkat cell lines deficient for caspase-8 or the adaptor FADD (Fas associated death domain) were not protected from DEM-induced apoptosis. Caspase-3 activation preceded that of caspase-9 and initial processing of caspase-3 was regulated independent of caspase-9 and Bcl-2. However, inhibitors of caspase-9 or caspase-6 regulated caspase-3 later in the pathway. We explored the mechanism by which caspase-3 processing is regulated in this system. DEM triggered a loss of Erk-1/2 phosphorylation and XIAP (X-linked inhibitor of apoptosis protein) expression. The phorbol ester PMA activated a MEK-dependent pathway to block caspase-3 processing and cell death. Constitutively active MEK-1 (CA-MEK) upregulated XIAP expression and exogenous XIAP inhibited DEM-induced apoptotic damage. Thus, we describe a pathway where caspase-3 functions to initiate apoptotic damage and caspase-9 and caspase-6 amplify the apoptotic cascade. Further, we show that MEK may regulate caspase-3 activation via the regulation of XIAP expression in these cells.

Keywords: apoptosis; Bcl-2; caspase-3; Erk; mitochondria; T cells.

Introduction

The pivotal role that caspases play in triggering apoptosis has been documented in many systems.[1] On the basis of substrate specificity and some functional overlap, the caspase family of proteases are broadly divided into three main groups.[2] These include, caspases involved in inflammatory responses (caspase-1, 4, 5 11 and 13), effector caspases (caspases 3, 6 and 7) which target the degradation of cellular proteins and apical or regulator caspases (which include caspases 8 and 9), that principally activate the more distal, effector caspases. There are two clearly defined pathways that lead to the activation of apoptotic caspase cascades in cells. Oligomerisation of transmembrane death receptors such as Fas or Tumor Necrosis Factor Receptor (TNFR) activate procaspase-8 through well characterized receptor complexes.[3] The other major pathway is non-receptor mediated and integrated by the mitochondrion.[4] Procaspase-9 released from the mitochondrion is activated via interactions with Apaf-1 and cytochrome c in the presence of dATP. Once activated, the apical caspases-8 or -9 cleave effector caspases-3, -6 or -7 to initiate caspase cascades[5,6] that may be insult specific[7] and are required for orderly damage associated with apoptotic death.[8]

Attempts to order the sequence of caspase cascades have yielded conflicting results regarding the activation of caspase-6 and caspase-3.[7,9,10] While most death pathways are initiated by caspase-8 or caspase-9, there is evidence that other caspases can function to initiate apoptotic cascades. Thus, caspase-9 carries a caspase-3 cleavage site[11] and can function as a substrate for caspase-3[5] and caspase-12.[12]

In this study, we show that caspase-3 initiates apoptosis triggered by the glutathione depleting agent diethylmaleate (DEM). Initial activation of caspase-3 is independent of caspase-9 and Bcl-2. We present evidence that apoptotic damage is regulated via extracellular signal-regulated kinase-1/2 (Erk)-dependent signaling that is likely disrupted by diethylmaleate (DEM) in these cells. XIAP (X-linked inhibitor of apoptosis protein) inhibited caspase-3 activation and apoptosis in DEM treated cells. Our experiments suggest that XIAP expression and caspase-3 activation are regulated by MEK signaling in these cells.

Materials and methods

Reagents and cells

Diethylmaleate (DEM), Hoechst 33342, propidium iodide (PI) and PMA were obtained from Sigma Chemical

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Co. (St. Louis, USA). Peptide inhibitors Z-Val-Ala-Asp (O-methyl)-fluoromethyl ketone (ZVAD-fmk), Ile-Glu-Thr-Asp-fmk (IETD-fmk), Val-Glu-Ile-Asp-FMK (VEID-fmk), Leu-Glu-His-Asp-fmk (LEHD-fmk) and Z-Phe-Ala-fmk (ZFA-fmk) were obtained from Enzyme Systems Products (Dublin, CA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazoleylcarbocyanine iodide (JC-1) and 3,3′-diethyloxacarbocyanine ($DiOC_6$), were obtained from Molecular Probes (Eugene, OR). Antibodies that recognize the cleaved forms of caspases-7 and -9, caspase-3, the phospho-isoform of Erk, XIAP and the reagent UO126 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies to Erk and p38MAPK were from Santa Cruz Biotechnology (Santa Cruz, USA) and the antibody to caspase-6 was obtained from BD Biosciences (California USA). The human leukemia cell line Jurkat and isogenic mutants deficient for FADD (I 1.2) or caspase-8 (I 9.2) were obtained from ATCC (Virginia, USA). Cells were routinely maintained in complete medium (RPMI-1640 supplemented with 10% heat inactivated FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin) and were usually between 0.4–0.5 $\times$ 10^6/ml when used in experiments.

Induction of cell death

Jurkat cells were cultured (0.4 $\times$ 10^6/ml) in the presence or absence of 0.4 mM DEM for varied lengths of time described in each experiment. When required, cells were preincubated with the various inhibitors for 45 minutes at 37°C before the addition of DEM. Cell lysis was assessed by flowcytometric analysis of propidium iodide uptake (50 ng/ml). DNA fragmentation was assayed by flowcytometry[13] and analyzed using CELL Quest software (FACScan®, Becton Dickinson). To assess apoptotic nuclear damage, cells were stained with 1 μg/ml Hoechst 33342 for 5 minutes at 37°C before analysis of nuclear morphology by fluorescence microscopy.

Mitochondrial transmembrane potential

Staining for $DiOC_6$ or JC-1 was performed as described.[13] Cells were protected from light at all stages of the staining protocol. At the end of incubation, cells were washed in excess PBS and resuspended in 0.3 ml PBS and analyzed flowcytometrically according to the manufacturers instructions.

Analysis of caspase 3-like activity in intact cells

Cell pellets were incubated at 37°C with 50 μl of the PhiPhiLux reagent and 5 μl FCS for 1 hour. At the end of incubation, cells were washed with excess complete medium and analyzed by flowcytometry for fluorescence derived from PhiPhiLux cleavage in PI negative cells.

Western blot analysis

Whole cell lysates were resolved on SDS gels and western blot analysis performed using standard protocols.[13] In experiments involving caspase-9 or XIAP analysis, cell lysates were prepared in a cell extract buffer according to the manufacturer's instructions (NEB, USA). Proteins were detected by chemiluminescence according to the manufacturer's instructions (Pierce, USA).

Plasmids

The constitutively active MEK-1 plasmid (pCHA-MEK1 S218/222D) was originally from the laboratory of M. J. Weber (University of Virginia, Health Sciences Center, Charlottesville, Virginia, USA) and was obtained from Shahid Jameel (International Center for Genetic Engineering and Biotechnology, New Delhi, India). The XIAP construct was a kind gift of Colin S. Duckett (University of Michigan Medical School, Ann Arbor, Michigan, USA). The caspase-9S plasmid was a generous gift of Peter Vandenabeele (Department of Molecular Biology, University of Gent and Flanders, Gent, Belgium).

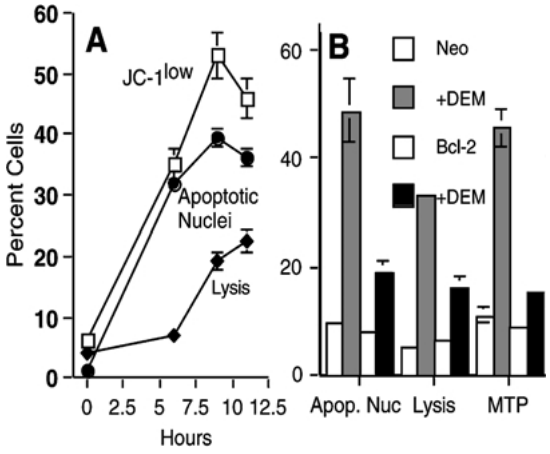
Transient transfections

5–10 $\times$ 10^6 cells were transfected with 1.5 μg pEGFP-N3 or combinations of 5 μg CA-MEK + 1.5 μg pEGFP-N3 or 3 μg XIAP + 1.5 μg pEGFP-N3 or 3 μg Bcl-2 + 1.5 μg pEGFP-N3 or 5 μg caspase-9S + 1.5 μg pEGFP-N3 plasmids by electroporation at 250 mV and 960 μF. Cells were cultured for 6–8 hours to allow expression of transfected genes and then continued in culture in the presence or absence of DEM. 14–16 hours after addition of DEM, cells were harvested and stained with H33342 as described above. GFP positive cells were viewed under a blue filter and nuclear morphology of GFP positive cells was scored using the UV filter. For the analysis of caspase-3 processing, transfected cells were analyzed 4–5 hours after the addition of DEM.

Results

Jurkat cells treated with diethylmaleate (DEM, 0.4 mM) undergo apoptotic death characterized by nuclear damage (Figure 1A, circles), loss of mitochondrial integrity/MTP (mitochondrial transmembrane potential) (Figure 1A, squares) and cell lysis by 14–18 hours (Figure 1A, diamonds). A substantial increase in nuclear damage was observed by at 6 hours. Approximately 8 hours after the addition of DEM, the nuclear fragments shrink to an extremely small, dot-like appearance and increasing numbers of cells appear as "ghosts". Since cell preparations are scored on the basis of nuclear morphology, ghost-like cells

Figure 1. DEM induced apoptosis in Jurkat cells. A, cells cultured with 0.4 mM DEM were analyzed for apoptotic nuclei (circles) or loss of mitochondrial integrity (squares) and cell lysis (diamonds) at various time points shown in the figure. B, Effect of Bcl-2 overexpression (black bars), on DEM induced nuclear damage (Apop. Nuc), cell lysis (Lysis) and mitochondrial damage (MTP) measured 14 hours after addition of DEM. Cells expressing the neomycin gene alone were used as controls (Neo, gray bars). For each condition, cells cultured in the absence of DEM are indicated by open bars.

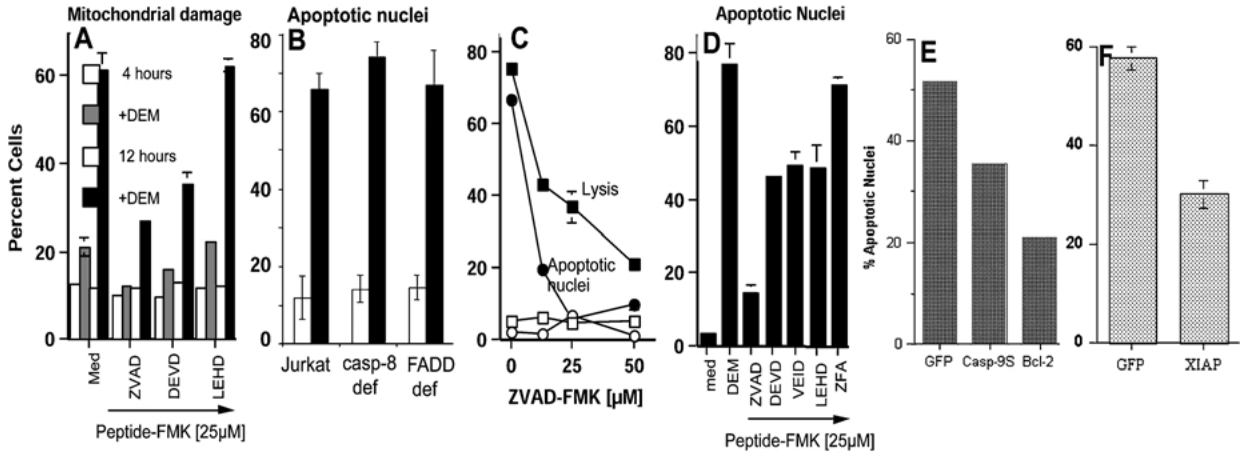


are not included in the analysis shown in Figure 1A. It is this exclusion that accounts for the apparent decrease in the number of cells with apoptotic nuclei over the duration of the assay. Cell lysis increased progressively to reach maximum levels 18–20 hours after addition of DEM (data not shown). Thus, like many other paradigms, loss of plasma membrane integrity was a late event and followed nuclear damage in the death pathway.

Since mitochondrial damage preceded cell lysis (Figure 1A, compare 35% cells with MTP loss vs. less than 5–7% lysis at the 6 hour time point), we tested the effect of the anti-apoptotic protein Bcl-2 on DEM induced apoptosis. Bcl-2 inhibits death by heterodimerizing with pro-apoptotic proteins such as Bax and t-Bid, thereby preventing the release of apoptogenic intermediates from mitochondria.[14] As shown in Figure 1B, constitutive expression of Bcl-2 (black bars) protected cells from DEM induced nuclear damage and loss of MTP, consistent with the role of mitochondria as a site of amplification of the death pathway. Cell lysis was not inhibited as effectively by Bcl-2 suggesting a possible extra-mitochondrial component in the lytic process. Gray bars represent conditions with cells stably transfected with the *neo* gene alone.

Mitochondrial release of cytochrome c triggers the processing and activation of procaspase-9 in the presence of Apaf-1 and dATP, thereby initiating a post-mitochondrial caspase cascade in cells.[15] To assess if caspases were responsible for mitochondrial damage we tested various peptide inhibitors for their effect on DEM-induced loss of mitochondrial transmembrane potential (Figure 2A). The broad spectrum caspase inhibitor ZVAD-fmk blunted the

Figure 2. Role of caspases in DEM induced apoptotic damage. A, Jurkat cells pre-treated with indicated peptides and cultured in the presence of DEM for 4 (gray bars) or 12 (black bars) hours were analyzed for loss of MTP at each time point. Open bars represent cells cultured without DEM at each time point. B, Jurkat cells, or the caspase-8 deficient (casp-8 def) or FADD deficient (FADD def) mutants were cultured with (black bars) or without (open bars) DEM. Cells were analyzed for apoptotic nuclear damage after 18 hours. C, Jurkat cells pre-treated with different concentrations of ZVAD-fmk shown in the figure were cultured with (filled symbols) or without (open symbols) DEM for 18 hours and analyzed for cell lysis and DNA fragmentation. D, Jurkat cells were pre-treated with 25 μM of the various peptides indicated in the figure and cultured with 0.4 mM DEM for 14–16 hours. Cells were analyzed for apoptotic nuclear damage. Data are a mean of four independent experiments. E, Cells were transfected with GFP alone or with GFP + Caspase-9S (casp-9S) or GFP + Bcl-2 as described in methods. Cells were scored for apoptotic nuclear damage 18 hours after the addition of DEM. The data shown in the panel are normalized to apoptotic damage in the absence of DEM. A representative experiment of three independent experiments has been shown. F, Cells were transfected with GFP or GFP + XIAP and treated exactly as described in E. All data is derived from three independent experiments unless stated otherwise.



loss of MTP. As shown in Figure 2A, when measured at 4 (gray bars) or 12 (black bars) hours after addition of DEM, the caspase-3/7 inhibitor DEVD-fmk blunted MTP loss whereas LEHD the peptide more specific for caspase-9 was without effect in this assay.

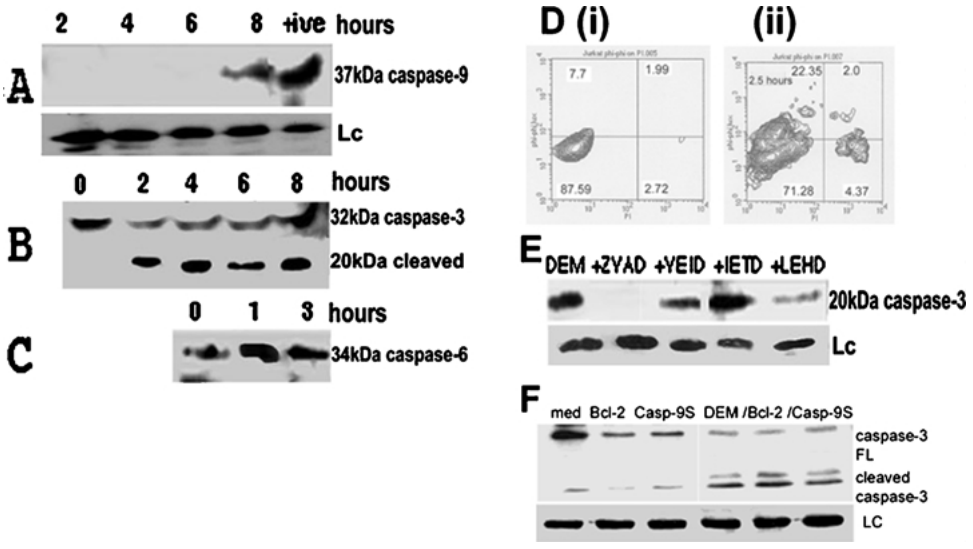
Since mitochondrial damage can be triggered by caspase-8 via the processing of the pro-apoptotic protein Bid, we tested Jurkat cell mutants with a deficiency for caspase-8 or FADD for their susceptibility to DEM-induced apoptosis. As shown in Figure 2B, the susceptibility of mutant cell lines to DEM mediated apoptotic damage was comparable to the parental cell line, arguing against a critical role for caspase-8 and perhaps receptor-dependent signaling in this pathway.

ZVAD-fmk blocked DEM triggered lysis (Figure 2C, squares), and apoptotic nuclear damage (Figure 2C, circles) to the level of untreated cells, suggesting a role for caspases in this pathway. The peptides DEVD-fmk, LEHD-fmk and VEID-fmk also reduced nuclear damage triggered by DEM treatment (Figure 2D). The peptide βZFA-fmk was without effect and served as the negative control in these experiments. DEM-induced apoptotic damage was also reduced in caspase-9S (dominant negative caspase-9) transduced cells (Figure 2E). Although, protection with caspase-9S was not as effective as that achieved by transfecting Bcl-2 the data with both genes indicated that a pre-mitochondrial event was probably initiating apoptotic damage. Further, overexpression of XIAP inhibited DEM-induced apoptotic damage consistent with a role for caspases in this pathway (Figure 2F).

We then assessed kinetics of caspase processing, particularly caspase-9 and caspase-3 in DEM treated cells. Unexpectedly, in enzymatic assays of caspase activity we could not detect caspase activity in DEM treated cells. DEM at the concentrations used in our assay system [0.4 mM] also decreased caspase protease activity when added into cell extracts prepared from Fas treated Jurkat cells [data not shown]. The negative modulation of caspase activity by redox reagents when present in cell extracts has been reported earlier for the thioloxidant diamide.[16] Therefore, we used western blot analysis to follow the processing of different caspases. An antibody that recognizes only the 37 kDa form of cleaved caspase-9 indicated that processing of this caspase occurred 8 hours after addition of DEM (Figure 3A). On the other hand, DEM triggered processing of caspase-3 was clearly detected two-four hours after addition of DEM (Figure 3B) and preceded the processing of caspase-9. Caspase-6 can activate caspase-3 in some systems,[9,10] but we did not detect any loss of the full length 34 kDa form of caspase-6 (Figure 3C), or caspase-7 (data not shown) at earlier time points.

To confirm the induction of caspase-3 activity early in the pathway we used the cell permeable reagent

Figure 3. Caspase activation in DEM treated cells. A–C, DEM treated Jurkat cells were analyzed, at the times indicated above the blots for the expression of 37 kDa cleaved caspase-9 (panel A), full length and the 20 kDa cleaved form of caspase-3 (panel B) and full length caspase-6 (panel C) by immunoblot analysis. Cell lysates supplied by the manufacturer (NEB, USA) were used as the positive control for cleaved caspase-9. D, Cells treated for 2.5 hours with (ii) or without (i) DEM were loaded with PhiPhiLux and analyzed by flowcytometry as described in methods. Populations in the upper left quadrant indicate live [PI-] cells with caspase-3-like activity. Dead cells positive for this activity are in the upper right quadrant. Cells in the lower-left quadrant are live cells with no detectable caspase-3-like activity by this assay. E, Jurkat cells were pre-treated with 25 μM of the various peptides and cultured for 8 hours with DEM. Cell lysates were analyzed by immunoblot analysis for the presence of cleaved caspase 3 in different conditions. The blot shows the presence of the 20 kDa cleaved product of caspase-3. LC indicates the loading control. F, Jurkat cells transfected with different genes were cultured with (lanes 4–6) or without (lanes 1–3) DEM as described in methods. After 5 hours cell lysates were analyzed for processing of caspase-3 by western blot analysis. LC indicates the loading control for parity of loading across different lanes.



PhiPhiLux-G_1D_2 substrate[7] to measure caspase-3/7 enzymatic activity in intact cells (Figure 3D). In 2.5 hours DEM treatment resulted in the appearance of a subset of cells with increased fluorescence (22.35% in the upper left quadrant) indicative of the activation of caspase-3 (panel ii). In control, untreated cells (panel i) at this time approximately 7.7% cells were positive for this activity. The number of cells positive for caspase-3 activity correlated with the apoptotic damage to DEM treated cells and increased progressively with time.

Since LEHD and VEID inhibited nuclear damage (Figure 2D) we assessed the effect of these peptides on the processing of caspase-3 late in the pathway. As shown in Figure 3E, when assessed 8 hours after the addition of DEM, ZVAD-fmk expectedly blocked caspase-3 activation (lane 2) whereas, IETD-fmk an inhibitor of caspase-8 did not block the appearance of the cleaved caspase-3 product (Figure 3E, lane 4). The cleaved form was lower in cells treated with peptide inhibitors of caspase-6/VEID (Figure 3E, lane 3) or caspase-9/LEHD (Figure 3E, lane 5). In the experiment shown in figure 3F we assessed caspase-3 processing in cells transfected with the dominant negative caspase-9 construct, caspase-9S,[17,18] or Bcl-2. DEM induced processing of caspase-3 at the 5 hour time point (lane 4) was not blocked by caspase-9S (lane 6) or Bcl-2 (lane 5). In fact, in Bcl-2 expressing cells we detect an increase in the accumulation of the cleaved caspase-3 product. From these experiments we concluded that caspase-3 processing was an early event in DEM induced apoptosis in Jurkat cells and its activation was regulated in part, by caspase-6 and caspase-9 only at the later stages of the apoptotic pathway.

Interactions amongst various caspases results in a network of activated caspases, and has been demonstrated using both recombinant proteins and intact cells.[5–7] There is not as much known about the initial events governing activation of effector caspases such as caspase-3 in the absence of apical caspase activation. We have recently shown that caspase-3 activity can be regulated by calpain, a calcium-activated protease in T cells.[19] However, cell permeable inhibitors of calpain did not block DEM induced apoptosis ruling out the possible involvement of calpain in this process (data not shown).

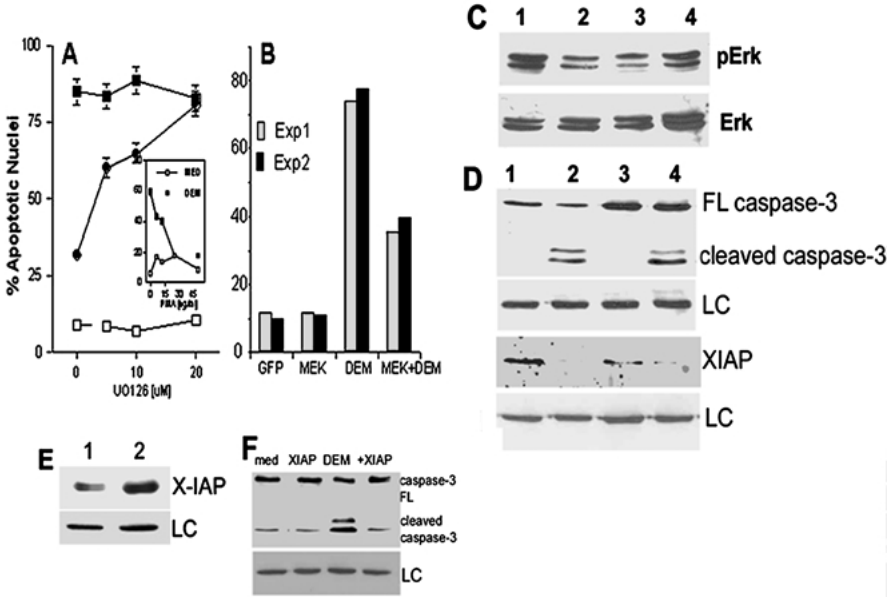
DEM-induced apoptosis was blocked by the phorbol ester PMA (Figure 4A, inset) and the protective effect of PMA was attenuated by UO126 (Figure 4A, filled circles), a pharmacological inhibitor of MEK.[20] At the concentrations tested, UO126 was not toxic to cells (Figure 4A, open squares) and did not increase DEM induced apoptosis in the absence of PMA (Figure 4A, filled squares). We also tested the effect of a constitutively active construct of MEK-1 (pCHA-MEK1 S218/222D) in this system. As shown in the two experiments in Figure 4B, constitutively active MEK-1 (CA-MEK) partially reduced DEM-induced apoptosis. In subsequent experiments we examined the effect of DEM on the extracellular signal-regulated kinase (Erk) phosphorylation as a first step towards the analysis of the mechanism by which MEK may regulate the caspase-3 dependent component of DEM induced apoptosis.

DEM triggered a progressive loss of phosphorylation of Erk in Jurkat cells (Figure 4C). The phosphorylation of Erk (pErk) was reduced in cells treated with DEM for 1 hour (lane 2) or 2.5 hours (lane 3) as compared to control, untreated cells (lane 4). There was some decrease in total Erk in the first 30 minutes (lane 1), but levels of Erk did not fall further after 1 hour. As shown in Figure 4D, DEM induced caspase-3 processing measured after 14 hours (lane 2), was blocked by PMA (lane 3). The inhibitory effect of PMA was abrogated by UO126 (lane 4). Further, as shown in the third blot in the series (Figure 4D), DEM triggered loss of expression of XIAP (lane 2) was restored by PMA (lane 3) via a UO126-sensitive mechanism (lane 4). Consistent with these data, over-expression of CA-MEK elevated expression of XIAP (Figure 4E, lane 2) as compared to mock transfected cells (Figure 4E, lane 1). DEM-induced processing of caspase-3 at an early time point (Figure 4F, lane 3) was blocked by the overexpression of XIAP (Figure 4F, lane 4). Transfected cells also showed a low level of spontaneous cleavage of caspase-3 (Figure 4F, lanes 1 and 2).

Discussion

Caspase activation is strongly cell-type and stimulus dependent. The experiments in this study support a role for caspase-3 as an initiator of DEM triggered apoptosis in Jurkat T cells. A DEM-induced apoptotic pathway sharing some, but not all the characteristics we describe here has been reported in prostate carcinoma cell lines.[21] Two major pathways for activation of caspase cascades that position caspase-8 or caspase-9 as apical events have been described.[1–4] Both these caspases target activation of caspase-3, for amplification of caspase cascades in cells.[22] However, DEM triggered caspase 3 cleavage preceded the activation of caspases-9, -6 (Figure 3), or -7 (data not shown). Processing of caspase-3 at earlier time points was not blocked by a dominant negative form of caspase-9 or Bcl-2 (Figure 3F). Both genes inhibited DEM-induced apoptotic damage (Figure 2E). Further, DEM triggered apoptosis was not dependent on caspase-8 or FADD arguing against caspase-8 or caspase-10 functioning as activators of caspase-3. Inhibition of caspase-3 blocked loss of MTP and nuclear damage whereas inhibition of caspase-6 or caspase-9 blocked nuclear damage alone. The functional involvement—indicated by dominant-negative constructs or peptide inhibitors—of caspases-6 and -9 suggest that they are probably activated as part of positive

Figure 4. Effect of MEK signaling on DEM-induced apoptosis. A, Jurkat cells were cultured in the presence of different concentrations of UO126 alone (open squares), UO126 + DEM (closed squares) or UO126 + DEM + 40 ng/ml PMA (closed circles) for 18 hours. At the end of culture cells were harvested and analyzed for apoptotic nuclear morphology as described in methods. Inset: dose-response effect of PMA on DEM-induced apoptosis (closed symbols). Open symbols represent cells cultured in the presence of PMA alone. B, Jurkat cells transfected with GFP alone or GFP + MEK-1 were cultured in the presence or absence of DEM and analysed for DNA damage as described in methods. Data from two independent experiments is shown. C, Total cell lysates of cells treated with DEM for 0.5 hours (lane 1), 1 hour (lane 2), 2.5 hours (lane 3) or cultured as such (lane 4) were assessed by western blot analysis for expression of pErk and then re-probed for Erk as described before.[29] D, Cell lysates prepared from cells cultured for 14 hours in the following conditions: cultured as such (lane 1), with DEM (lane 2), or with DEM + PMA (lane 3), or with DEM + PMA + UO126 (lane 4). Proteins were resolved by gel electrophoresis and membranes probed sequentially for the indicated proteins by western blot analysis. p38MAPK was used as the loading control (LC). E, Lysates prepared from cells 16 hours after transfection with GFP alone (lane 1) or GFP + MEK-1 (lane 2) were probed for expression of endogenous XIAP by western blot analysis. p38MAPK served as the loading control (LC). F, Jurkat cells transfected with GFP (lanes 1 and 3) or GFP + XIAP (lanes 2 and 4) were treated with DEM (lanes 3 and 4) or cultured as such (lanes 1 and 2) as described in methods. 5 hours after addition of DEM cell lysates were prepared from the different transfection groups and analyzed for the processing of caspase-3.



feed-back loops initiated by caspase-3. This would also account for the regulation of cleaved caspase-3 levels by inhibitors of caspase-6 and -9 (Figure 3E) at later stages (8 hours) in this pathway.

The unusual role of caspase-3 as an initiator caspase has been observed in other systems[23–26] and it appears that the mechanism of caspase-3 activation, in the absence of apical caspase activation, may vary in different systems. Changes in intracellular acidification regulate caspase-3 activation as a post-mitochondrial event in apoptotic death pathways.[27] Caspase activation can also be initiated by other cytoplasmic proteases such as the Ca^{++} activated protease calpain,[19,28] although DEM induced apoptosis was not blocked by calpain inhibitors in Jurkat cells.

Kinases have provided a link between extracellular survival signals and cellular apoptotic machinery and the MEK pathway has been previously implicated in protection from various apoptotic signals.[29–34] The mechanisms range from transcriptional or post-translational regulation of proteins of the Bcl-2 family,[30,31] phosphorylation dependent modification of pro-apoptotic molecules[29,32] or the inhibition of apoptotic events post cytochrome c release.[33,34] We propose a role for Erk in the regulation of DEM-induced apoptosis based on the following observations: (1) CA-MEK inhibited DEM-induced apoptosis, (2) inhibition of apoptosis by the phorbol ester PMA was abrogated by a pharmacological inhibitor of MEK, (3) DEM disrupted phosphorylation of endogenous Erk in cells and (4) the inhibition of caspase-3 processing by PMA was attenuated by the MEK inhibitor. MEK regulation of DEM-induced apoptosis is not restricted to the Jurkat cell line. The d11S T-cell hybridoma has relatively high levels of endogenous Erk-1/2[29] and is resistant to DEM-induced apoptosis. In these cells, inhibition of Erk by a dominant negative MEK construct or UO126 revealed a DEM-induced caspase-3 dependent apoptotic pathway (data not shown). These data suggest that MEK regulation of this pathway at the level of caspase activation may be a more general event.

Our experiments indicate that sustained activation of Erk abrogates the activation of caspase-3 (Figure 4D). This event is likely an indirect effect since typical Erk

phosphorylation sites are not seen on caspase-3. Therefore, we looked for an Erk-dependent mechanism that regulated caspase-3 processing.

Caspase activity is most often restrained by members of the IAP (inhibitors of apoptosis proteins) family of anti-apoptotic proteins which are found in a broad range of organisms[35] and inhibit caspase function by blocking substrate access to the binding/reactive site of the caspase.[36] Unlike the Bcl-2 family, IAPs block apoptosis by directly binding and inhibiting caspases. Since XIAP inhibits caspases-3, 7 and 9 we examined its modulation in this system. Overexpression of XIAP inhibited DEM-induced apoptotic damage and blocked the activation of caspase-3 in DEM treated cells. DEM reduced endogenous XIAP levels but the expression of this protein was restored in cells treated with PMA or elevated by over-expressing CA-MEK-1. Two other studies have shown that PKC activation via phorbol esters culminates in MEK-1/2 activation in HL-60, a human leukemia cell line.[37,38] In these studies, MEK blocked apoptosis triggered by singlet oxygen or taxol in these cells. It remains to be determined if the IAP are involved in conferring resistance to apoptosis in these systems.

Taken together, the data suggest that regulation of caspase-3 processing by Erk is probably mediated via IAP and may be a critical step in regulating susceptibility to apoptosis. Our data suggests that caspase-3 activates a pre-mitochondrial event in DEM treated cells that culminates in the loss of MTP. The molecular nature of the signal that disrupts the XIAP-mediated restraint of caspase-3 awaits identification. Ongoing experiments are attempting to identify molecules that associate with caspase-3/XIAP in DEM treated cells.

Conclusions

This study describes a paradigm where apoptosis is initiated by the classical effector caspase-3 which regulated both nuclear and mitochondrial damage triggered by DEM in a human leukemia cell line. We show that early activation of caspase-3 is independent of caspase-9 and Bcl-2. Caspase-9 functions to amplify the cascade as a caspase-9 blocking peptide or a dominant-negative form of caspase-9 partially inhibited nuclear damage and inhibited the processing of caspase-3 late in the death pathway. DEM-induced apoptosis was not dependent on caspase-8 or FADD-dependent transmembrane death receptor signaling. Apoptosis and caspase-3 processing were blocked by XIAP which was regulated, in turn, by MEK-dependent signaling.

Acknowledgments

The PhiPhiLux reagent was a generous gift of Beverly Z. Packard (Oncolummin Incorporated, Gaithersburg, USA) and is gratefully acknowledged. We acknowledge generous gifts of constructs from Colin S. Duckett and Peter Vandenabeele. We thank Neha Parikh and Hadassah Sade for their comments on the manuscript.

The study was funded in part by a grant from the Department of Science and Technology, Govt. of India and a core grant from NCBS to AS.

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