



# Commitment to cell death measured by loss of clonogenicity is separable from the appearance of apoptotic markers

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## Abstract

Kinetic analysis of dexamethasone-induced apoptosis in the human lymphoblastoid cell line CCRF CEM C7A has revealed a point when cells, morphologically indistinguishable from untreated cells, have irreversibly engaged a program leading to death, measured by a loss of clonogenicity. Since all cells that fail to clone eventually died through apoptosis, measurements of clonogenicity in this system provide an accurate measure of commitment to apoptotic death. Inhibition of caspases by peptide inhibitors blocked proteolysis of endogenous substrates and reduced nuclear condensation yet did not alter either dexamethasone-induced changes in clonogenicity or mitochondrial membrane potential. In contrast to the results with caspase inhibitors, expression of BCL-2 in CCRF CEM C7A cells proved sufficient to block all changes associated with apoptosis, including loss of both clonogenicity and changes in mitochondrial membrane potential. These results demonstrate that commitment to cell death can precede the key biochemical or morphological features of apoptosis by several hours and indicate that separate regulators govern cellular commitment to clonogenic death and the subsequent execution phase characterised as apoptosis.

**Keywords:** apoptosis; cell death; caspases; clonogenic; BCL-2

**Abbreviations:** DEX, dexamethasone; ICE, interleukin 1 $\beta$ -converting enzyme; PARP, poly (ADP-ribose) polymerase; AIP, apoptosis inducing factor; PVDF, polyvinylidene difluoride

## Introduction

Although apoptotic cells are characterized by a variety of morphological and biochemical markers (Cohen, 1993; Wyllie *et al*, 1980), analysis of the early events which lead to commitment to cell death has been hampered by the lack of features which unambiguously identify cells about to die. The study of the early stages leading to apoptosis, which we will refer to as *commitment*, is also complicated by the general asynchronous nature of apoptosis. The human lymphoblastoid cell line CCRF CEM C7A (referred to hereafter as CEM C7A) is an attractive model for investigating early events in apoptosis since the glucocorticoid dexamethasone (DEX) initiates a relatively precise and synchronous program of cell death (Norman and Thompson, 1977; Wood *et al*, 1994; Yuh and Thompson, 1989). Glucocorticoid induced apoptosis in CEM C7A cells consists of a *priming* phase of 24–36 h during which there is a requirement for both the continual presence of drug and changes in gene expression. This is followed by an *execution* phase which is not dependent on either presence of drug or protein synthesis (Wood *et al*, 1994).

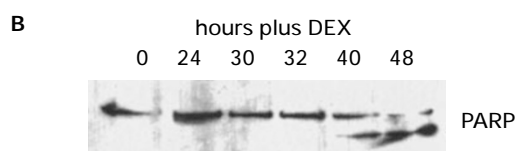
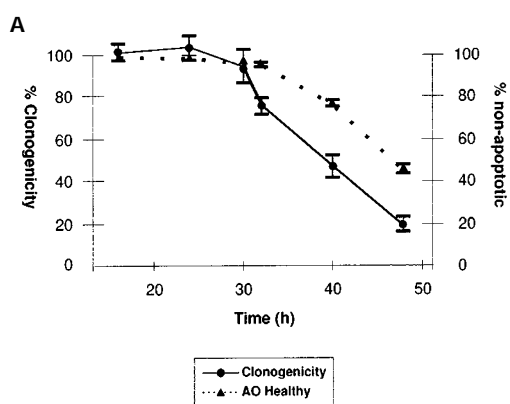
A major challenge in this and other cellular models of cell death is to ascertain the exact sequence of molecular and cellular events that lead to irreversible commitment to death and the final appearance of an apoptotic cell. Genetic studies of the nematode *C. elegans* have provided a model of apoptosis in which the *ced-9* gene, a homologue of the mammalian *BCL-2* gene, is upstream of *ced-3* gene, a protease related to the mammalian interleukin 1 $\beta$ -converting enzyme (ICE) (Shaham and Horvitz, 1996). Activation of the *Ced-3/ICE*-related proteases (caspases) (Alnemri *et al*, 1996) occurs in many cells as apoptosis is engaged (Fernandes-Alnemri *et al*, 1994, 1995; Nicholson *et al*, 1995). It has been suggested that activation of these proteases is required (Enari *et al*, 1995; Los *et al*, 1995) and possibly sufficient to induce apoptosis (Fernandes-Alnemri *et al*, 1995; Lazebnik *et al*, 1994; Schlegel *et al*, 1996). Although inhibition of caspases in intact cells is sufficient to prevent commitment to apo1/fas-mediated cell death (Geley *et al*, 1997a; Longthorne and Williams, 1997), death induced by other agents, including glucocorticoids, is not altered by caspase inhibition alone and appears to be regulated by *BCL-2* and related family members (Geley *et al*, 1997a, b; McCarthy *et al*, 1997; Xiang *et al*, 1996; Yang and Korsmeyer, 1996). Although both inhibition of caspases and expression of *BCL-2* are capable of blocking aspects of cell death it is not yet clear which phase(s) of glucocorticoid induced CEM C7A cell death they affect. We demonstrate here that through measuring clonogenicity we can unambiguously identify cells committed to subsequent apoptotic death. Using this assay we show that commitment to cell death occurs prior to loss of mitochondrial

membrane potential, chromatin condensation, or protein or DNA cleavage. Furthermore, we demonstrate that BCL-2 is able to block both commitment to cell death and the appearance of apoptosis whereas inhibition of caspases retards the biochemical and morphological markers of apoptosis but does not alter commitment to cell death.

## Results

### Kinetic analysis of morphological death and clonogenicity

To identify early stages leading to apoptosis, the kinetics of the appearance of cells with condensed chromatin was compared to the irreversible loss of proliferative capacity measured by a clonogenicity assay (Figure 1A). In the clonogenic assay sampled cells were diluted free of DEX and plated into 96 well plates at densities of between 0.33 and 10 cells per well under conditions that allow all individual untreated cells to form a colony. For time points preceding and including 30 h of DEX treatment, both morphological and clonogenic assays indicated essentially 100% viability. However, after a total of 32 h of DEX treatment there was a significant drop in clonogenicity in the absence of a detectable change in the frequency of cells exhibiting an apoptotic nuclear morphology (Figure 1A). This



**Figure 1** Kinetics of DEX-induced apoptosis, loss in clonogenicity and PARP cleavage. **(A)** Both clonogenicity and apoptosis were measured in CCRF CEM C7A cells treated with 5  $\mu$ M DEX at the indicated time points. Non-apoptotic cells were determined by staining cells with acridine orange and apoptotic cells were counted on the basis of either condensed or fragmented chromatin. An example of a typical non-apoptotic nuclei is shown in Figure 3A labelled NO DRUG and examples of typical condensed and fragmented chromatin are labelled as zVAD-fmk/DEX and DEX respectively. Over 200 cells were counted in each sample. Clonogenicity was determined by plating cells in 96 well plates to a final density of 0.33 or 10 cells/well. After two weeks incubation wells containing clones were scored and clonogenicity was calculated as described in the text. Results are expressed as the mean  $\pm$  S.E.M. from at least three experiments. **(B)** PARP cleavage in CCRF CEM C7A cells incubated with DEX for the indicated times was determined by immunoblotting as described in the text

difference in morphological and clonogenic death persisted in all samples treated for longer than 30 h, with loss of clonogenicity consistently exceeding the percentage of cells exhibiting an apoptotic morphology (Figure 1A). A comparison of the kinetics of clonogenic and morphological death under these conditions established that a minimum of 32 h of DEX treatment was required for significant commitment to death. Once committed, cells required up to 8 h to display the morphological features of apoptosis.

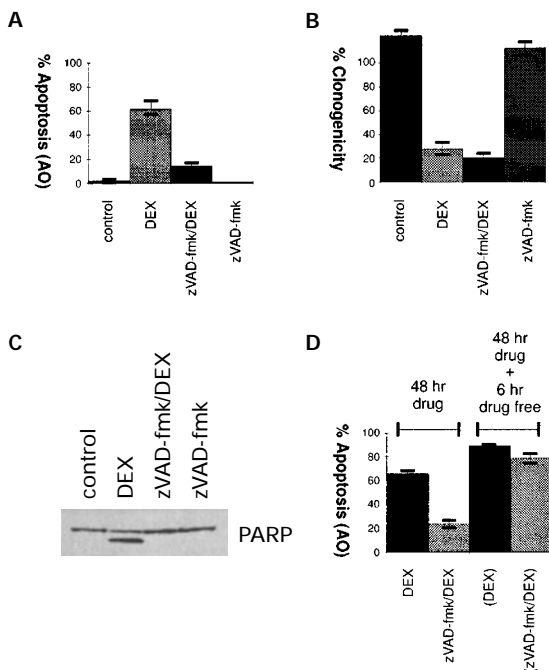
### Caspase activity detectable after commitment to apoptotic death

Caspases, such as CPP32 are activated during apoptosis in many systems (Fernandes-Alnemri *et al*, 1994, 1995; Nicholson *et al*, 1995). Furthermore, activation of these proteases has been shown to be required (Enari *et al*, 1995; Jacobsen *et al*, 1996; Los *et al*, 1995) and possibly sufficient for the induction of apoptosis (Fernandes-Alnemri *et al*, 1995; Lazebnik *et al*, 1994; Schlegel *et al*, 1996) although it is not clear which of the caspases are essential for apoptosis (Kuida *et al*, 1996). To determine whether these proteases were involved in commitment to clonogenic cell death, the kinetics of PARP cleavage was determined by immunoblotting and compared to changes in clonogenicity and nuclear morphology. After 32 h of exposure to DEX when over 20% of cells were unable to form clones, and less than 5% of cells showed chromatin condensation (Figure 1A) PARP cleavage was not detectable (Figure 1B). PARP cleavage was first detected in cells treated with DEX for 40 h (Figure 1B), a point when over 20% of cells were clearly morphologically apoptotic judged by chromatin condensation (Figure 1A). These results suggest that caspases capable of PARP cleavage are active only after commitment to death has occurred and that they are associated with the appearance of apoptosis.

### Inhibition of caspase activity does not affect commitment

To further examine whether the caspases were involved in commitment to death we examined the effect of short peptide inhibitors of caspases known to act on intact cells (An and Knox, 1996; Jacobsen *et al*, 1996; Nicholson *et al*, 1995). CEM C7A cells were treated with DEX and/or the peptide inhibitor zVAD-fmk (50  $\mu$ M). Immunoblotting revealed that zVAD-fmk completely inhibited DEX-induced PARP cleavage (Figure 2C) and morphological assessment revealed a large reduction in the number of cells undergoing chromatin condensation or cleavage (Figure 2A). However, loss of clonogenicity following exposure to DEX was unaffected by the presence of zVAD-fmk (Figure 2B) suggesting that although the inhibitor reduced morphological changes associated with apoptosis it did not alter commitment to death. Similar results were also seen with the CPP32 specific inhibitor Ac-DEVD-CHO which was able to inhibit over 90% of PARP cleavage in DEX treated CEMC7A cells but was unable to prevent clonogenic loss (unpublished data). We next examined whether some of the apparently healthy cells present in cultures treated with either DEX alone or DEX plus zVAD-fmk were already committed to apoptotic death. To measure commitment apoptosis was assessed by

acridine orange staining after 48 h treatment with DEX alone or DEX plus zVAD-fmk and also after a further 6 h incubation of treated cells in fresh media containing neither DEX nor zVAD-fmk (Figure 2D). The increase in apoptotic cells seen after washing DEX treated cells free of drug (compare the first and third columns in Figure 2D) is in agreement with previous results (Wood *et al*, 1994) and is consistent with the kinetic studies indicating that cells which are non-clonogenic eventually undergo chromatin changes (Figure 1A). The dramatic increase in apoptotic cells seen after washing DEX/zVAD-fmk treated cells free of drug and inhibitor (compare the second and fourth columns in Figure 2D) shows that the majority of apparently healthy cells present after 48 h treatment with DEX plus zVAD-fmk were already committed to apoptotic death. The combined data presented in Figure 2 together with the kinetics of protease activation (Figure 1B) strongly suggests that caspases are not required for commitment to death but are involved during the later execution phase of apoptosis.



**Figure 2** Commitment to clonogenic death is not affected by the caspase inhibitor zVAD-fmk. (A) Apoptosis in CCRF CEM C7A cells treated for 48 h with the indicated reagents was determined by staining with acridine orange as described for Figure 1. (B) Clonogenicity in CCRF CEM C7A cells treated for 48 h with the indicated reagents was determined by plating cells in 96 well plates to a final density of 0.33 or 3.33 cells/well as described in the text. (C) PARP cleavage in CCRF CEM C7A cells treated for 48 h with the indicated reagents was assessed by immunoblotting. Results are representative of three independent experiments. (D) Commitment to apoptosis was assessed by examining CCRF CEM C7A cells cultured in the presence of DEX or DEX plus zVAD-fmk. After 48 h incubation a fraction of the treated cells was assessed for visible apoptosis by staining with  $5 \mu\text{g ml}^{-1}$  acridine orange (columns 1 and 2) and the remaining cells washed twice, resuspended in fresh medium and incubated for a further 6 h and re-assessed for apoptosis (columns 3 and 4). Results are expressed as the mean  $\pm$  S.E.M. from three experiments. The concentrations used in all experiments were  $5 \mu\text{M}$  DEX and  $50 \mu\text{M}$  zVAD-fmk and controls received solvent alone

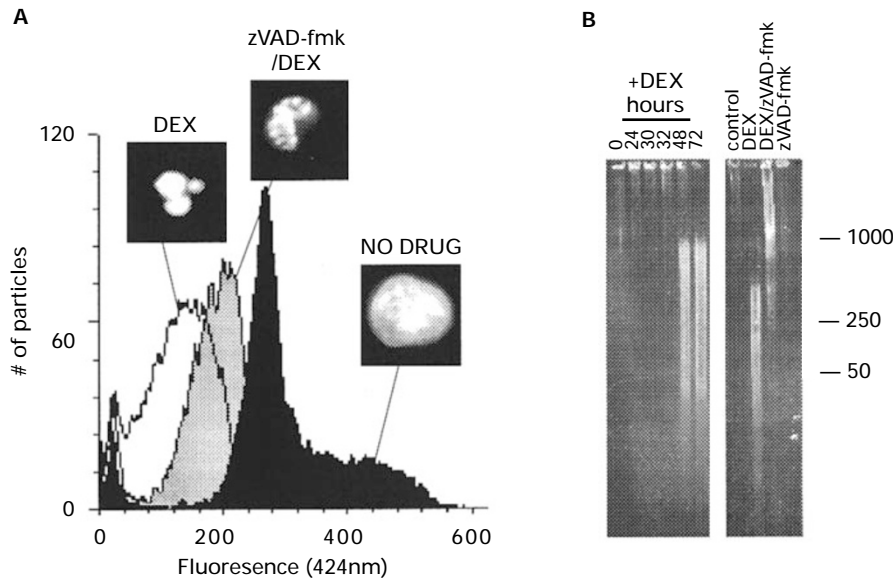
### Caspase inhibitor zVAD-fmk retards apoptotic chromatin and cytoplasmic changes

In comparing the morphology of untreated cells with cells treated either with DEX alone or with DEX plus zVAD-fmk for prolonged periods of time it was apparent that caspase inhibition did not block all cellular and nuclear morphological changes associated with apoptosis. For a more detailed evaluation, DEX and DEX plus zVAD-fmk treated cells were analyzed by fluorescence microscopy, flow cytometry, transmission electron microscopy and their DNA examined by gel electrophoresis. Microscopic and flow cytometric analysis of Hoechst 33342 stained CEM C7A cells grown in the presence of DEX for 72 h revealed the expected DEX-induced chromatin condensation/fragmentation and the development of a sub-G1 peak indicative of the presence of apoptotic cells (Figure 3A). Microscopic analysis of cells grown in the presence of DEX plus zVAD-fmk for 72 h revealed an accumulation of cells exhibiting condensed chromatin but with few, if any, cells containing fragmented nuclei (Figure 3A insets). Flow cytometric analysis of cells treated with DEX plus zVAD-fmk for 72 h revealed a sub G1 peak which was distinct from that seen with DEX alone (Figure 3A). Pulse field gel electrophoresis (PFGE) examination of DNA extracted from cells treated with DEX alone or with DEX plus zVAD-fmk revealed that DNA cleavage was apparent after commitment to death and that zVAD-fmk retarded DNA cleavage (Figure 3B) although it had no effect on the fall in clonogenicity.

To examine the overall morphology of cells treated with DEX plus zVAD-fmk, sections of untreated cells, cells treated with DEX alone and DEX plus zVAD-fmk were prepared and analyzed by transmission electron microscopy. A comparison of untreated and DEX treated cells revealed DEX-induced morphological changes typical of apoptosis including cell shrinkage, condensation/fragmentation of the nucleus and destruction of cytoplasmic organelles (Figure 4). The morphology of DEX plus zVAD-fmk treated cells suggested partial or arrested apoptosis with condensed yet intact chromatin and recognisable although abnormal mitochondria (Figure 4).

### Loss of mitochondrial membrane potential ( $\Delta\Psi$ ) occurs after commitment to death and is unaffected by addition of zVAD-fmk

The morphology of DEX plus zVAD-fmk treated cells suggested a partial condensation of chromatin yet intact DNA and recognisable, although abnormal, mitochondria (Figure 4). To examine whether zVAD-fmk altered the apoptosis-associated fall in mitochondrial  $\Delta\Psi$  (Zamzami *et al*, 1995) we examined mitochondrial  $\Delta\Psi$  in cells treated with DEX alone or DEX plus zVAD-fmk. Figure 5 shows that although addition of zVAD-fmk to DEX treated cells impaired chromatin condensation and completely inhibited PARP cleavage (Figures 2, 3 and 4) it did not alter the apoptosis associated fall in mitochondrial membrane potential. Since both loss of clonogenicity and mitochondrial  $\Delta\Psi$  are unaffected by addition of zVAD-fmk it is possible, as has been suggested (Susin *et al*, 1996), that loss of mitochondrial



**Figure 3** Inhibition of caspases by zVAD-fmk impairs chromatin changes in DEX treated cells. **(A)** CCRF CEM C7A cells were treated for 72 h with either 5  $\mu$ M DEX, 50  $\mu$ M zVAD-fmk, 5  $\mu$ M DEX and 50  $\mu$ M zVAD-fmk together or with solvents in the control sample. Nuclear morphology was examined by staining cells, fixed in 2% formaldehyde, with 811  $\mu$ M Hoechst 33342 and viewing using fluorescence-microscopy ( $\times 1000$  magnification). Changes in chromatin condensation were determined by staining with Hoechst 33342 and analysis by flow cytometry. Similar results were seen in two additional experiments. **(B)** DNA fragmentation was assessed by FIGE as described in the text. Samples from cells grown in the presence of 5  $\mu$ M DEX for the indicated period of time are shown in the left panel and samples prepared from CCRF CEM C7A cells treated with solvent (control), 5  $\mu$ M DEX, 50  $\mu$ M zVAD-fmk or 5  $\mu$ M DEX and 50  $\mu$ M zVAD-fmk together for 72 h are shown in the right hand panel. The position of 50, 250 and 1000 kilobase DNA size markers are shown to the right of the figure. Similar results were seen in two additional experiments

potential is the biochemical trigger responsible for clonogenic death. However, a comparison of clonogenicity and mitochondrial  $\Delta\Psi$  after DEX treatment (Figure 5B) revealed that the number of cells with normal mitochondrial  $\Delta\Psi$  was at least four times higher than the number of surviving clonogenic cells leading to the conclusion that the majority of cells with normal mitochondrial  $\Delta\Psi$  are already committed to death. We also noted that after DEX treatment there was a small yet reproducible excess of number of cells with morphologically normal nuclei compared to the number of cells with normal levels of mitochondrial  $\Delta\Psi$  suggesting that chromatin condensation occurs shortly after a drop in mitochondrial  $\Delta\Psi$ . Addition of zVAD-fmk to DEX treated cells did not significantly alter either clonogenicity or loss of mitochondrial  $\Delta\Psi$  when compared to DEX alone whereas it significantly reduced apoptosis measured by acridine orange staining (Figure 5B). Together the data presented in Figure 5 show that although loss of mitochondrial  $\Delta\Psi$  is not directly responsible for commitment to death in DEX treated CEM C7A cells it does not appear to be regulated by those caspases which are sensitive to zVAD-fmk.

### BCL-2 expression does not block DEX induced loss of proliferation but does prevent commitment to apoptosis

BCL-2 is the quintessential apoptosis suppressor gene. However, there are conflicting reports as to whether it can influence commitment to clonogenic death (Hashimoto *et al*,

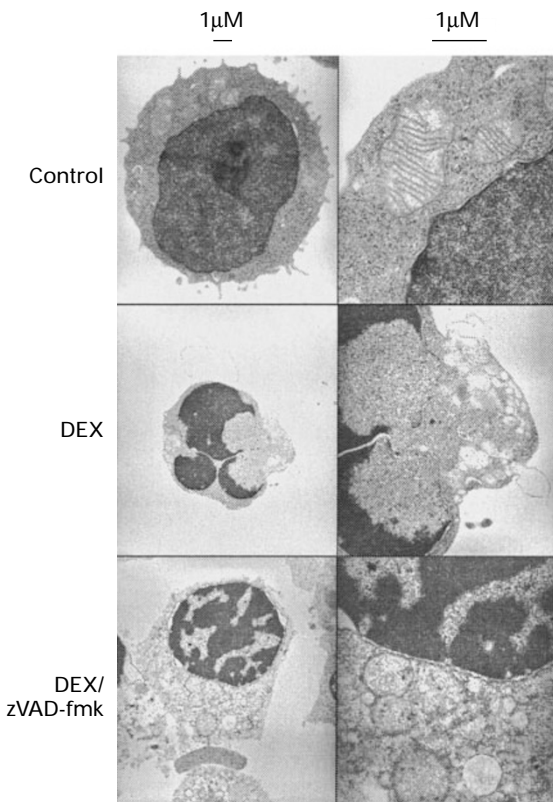
1995; Kamesaki *et al*, 1993; Lock and Stribinskiene, 1996; Strasser *et al*, 1994; Walker *et al*, 1997; Yin and Schimke, 1995). To assess whether BCL-2 is able to preserve clonogenicity after DEX treatment we compared established CEM C7A clones that have been previously transfected with a retroviral vector expressing BCL-2 (pZipBcl-2) or vector alone (pZipNeo) (Martin *et al*, 1995). Although the untreated BCL-2 transfected cells show an overall lower cloning potential than the stock CEM C7A cells it is clear that, unlike the addition of zVAD-fmk which protects from morphological but not clonogenic death, BCL-2 is extremely effective in blocking both forms of death (Figure 6A and B). Although DEX treated BCL-2 expressing cells fail to die, they nevertheless undergo DEX-induced cell shrinkage (R Benson *et al*. in preparation) and short term loss of proliferation (data not shown) indicating that BCL-2 specifically blocks commitment to death rather than general DEX responsiveness. The results with BCL-2 confirm that loss of clonogenicity in DEX treated CEM C7A cells is associated with commitment to death and are in agreement with recent results suggesting that BCL-2/BAX acts early in commitment to cell death and that they interact with a protease independent regulator(s) of cell death (McCarthy *et al*, 1997; Xiang *et al*, 1996).

### Discussion

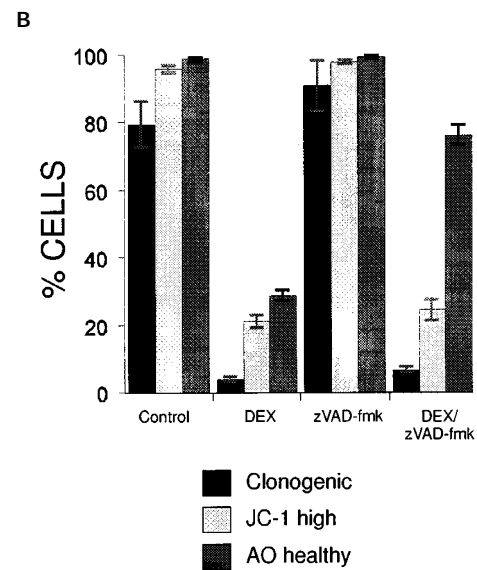
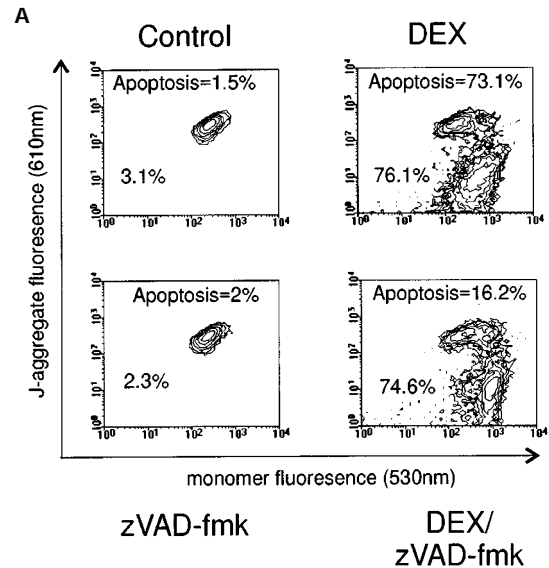
Apoptosis is a morphological description of a conserved type of cell death (Wyllie *et al*, 1980). However, it is not clear if any of the biochemical events associated with apoptosis, such as proteolysis, are critical for the irreversible commitment of a cell

to death. Addition of the caspase inhibitor zVAD-fmk to CEM C7A cells undergoing apoptosis in response to DEX blocked the cleavage of PARP (Figure 2C) and profoundly delayed chromatin alterations associated with apoptosis (Figures 2A, 3 and 4). The finding that zVAD-fmk efficiently blocked nuclear fragmentation of DEX treated CEM C7A cells is in agreement with a number of previous observations in other cell types (An and Knox, 1996; Jacobsen *et al*, 1996; McCarthy *et al*, 1997; Nicholson *et al*, 1995; Xiang *et al*, 1996). Despite the obvious potency of zVAD-fmk, it had no measurable effect on either the loss of clonogenic potential (Figure 2B) or the collapse in mitochondrial membrane potential induced by DEX treatment (Figure 5). Although we cannot rule out that concentration of zVAD-fmk used in Figure 2B (50  $\mu$ M) was not sufficient for complete inhibition of endogenous caspases we were unable to bring about any significant difference in clonogenicity by either repeated addition of zVAD-fmk or by increasing zVAD-fmk concentrations up to 200  $\mu$ M (unpublished data). These results are consistent with recent results using video microscopy to demonstrate that the onset of death in fibroblasts, apparent as membrane blebbing, is unaffected by zVAD-fmk whereas later execution stages are retarded in much the same manner as we have reported for CEM C7A cells (McCarthy *et al*, 1997). Although we have been unable to observe an early

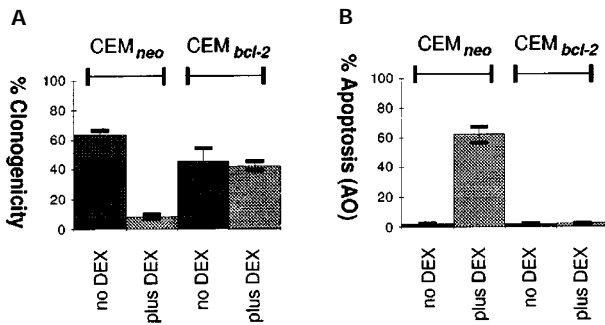
morphological change in CEM C7A cells equivalent to fibroblast membrane blebbing, the early loss of clonogenicity which is unaffected by zVAD-fmk suggests that, as was seen with fibroblast death (McCarthy *et al*, 1997), CEM C7A



**Figure 4** Ultrastructural changes in cells treated with DEX alone and DEX plus the caspase inhibitor zVAD-fmk. Ultrastructural analysis by transmission electron microscopy of CCRF CEM C7A cells treated for 48 h with either 5  $\mu$ M DEX, 5  $\mu$ M DEX plus 50  $\mu$ M zVAD-fmk or with solvents (control). Cells treated with zVAD-fmk alone were indistinguishable from controls (not shown)



**Figure 5** DEX-induced loss of mitochondrial membrane potential is not blocked by the caspase inhibitor zVAD-fmk. (A) Cells were analyzed as described and the resulting flow cytometry analysis of JC-1 staining are shown for cells treated for 48 h with either 5  $\mu$ M DEX, 50  $\mu$ M zVAD-fmk, 5  $\mu$ M DEX plus 50  $\mu$ M zVAD-fmk, or solvents (control). Loss of membrane potential in individual cells was determined by a fall in J-aggregate fluorescence (610 nm) and the percentage shown in the lower left of each graph. The percentage of apoptotic cells was also measured for each sample by acridine orange (AO) staining and indicated within each graph. (B) Clonogenic (black bars) and non-apoptotic (dark grey) measurements of cells treated for 48 h with either 5  $\mu$ M DEX (DEX), 50  $\mu$ M zVAD-fmk (zVAD-fmk), 5  $\mu$ M DEX plus 50  $\mu$ M zVAD-fmk (DEX/zVAD-fmk) or solvents (Control) were made as described for Figure 1. JC-1 high measurements are a result of flow cytometry analysis and indicate the percentage of cells with JC-1 staining comparable to levels seen in the control cells shown in A. Results are expressed as the mean  $\pm$  S.E.M. from three experiments



**Figure 6** Bcl-2 prevents DEX-induced apoptosis and loss in clonogenicity. (A) CEM<sub>neo</sub> and CEM<sub>bcl-2</sub> cells were cultured continuously for 72 h in the presence of 5  $\mu$ M DEX or an equivalent volume of solvent (no DEX). Clonogenicity was assessed by plating cells at a density of 1 or 10 cells per well in a 96 well. The slightly lower clonogenicity noted for the neo and bcl-2 clones compared to the levels seen with the stock CEM C7A most likely represents a minor, yet consistent, over-estimation of cell number in this set of experiments due to the use of a Coulter counter rather than the microscope to measure cell numbers. A repeat of clonogenicity experiments with neo and bcl-2 clones using microscopic evaluation of cell number resulted in 100% clonogenicity for untreated controls and the DEX treated bcl-2 clone (L Dubrez personal communication). (B) From the same cultures assessed for clonogenicity apoptosis was determined by staining the cells with acridine orange. All results are expressed as the mean  $\pm$  S.E.M. from four experiments

commitment to death in response to DEX precedes caspase activation and is separable from caspase activation. In contrast to DEX-induced death, all aspects of apo1/fas-induced death in CEM C7A cells, including clonogenicity, are affected by the addition of zVAD-fmk (Longthorne and Williams, 1997). Since DEX addition does eventually lead to the activation of caspases which can be inhibited by zVAD-fmk the observed loss of clonogenicity in the presence of DEX and zVAD-fmk (Figure 2) suggests that, unlike apo1/fas, DEX may activate either proteases unaffected by zVAD-fmk or events upstream of caspase activation. Additional components of a death pathway induced by DEX are also implied by the observations that loss of ICE activity through either gene deletion or introduction of the viral ICE inhibitor *crmA* leads to reduced apo1/fas apoptosis but does not affect glucocorticoid-induced death (Geley *et al*, 1997a; Kuida *et al*, 1995; Li *et al*, 1995).

In contrast to the partial inhibition of the morphology of apoptosis by zVAD-fmk and its complete failure to prevent a loss of clonogenicity, expression of BCL-2 blocks all aspects of death including both clonogenic and morphological death measured within 72 h of DEX addition (Figure 6). Xiang *et al* have also recently reported that zVAD-fmk does not completely block BAX-induced cell death in Jurkat cells and have proposed the existence of a death induction pathway regulated by BCL-2/BAX which is independent of caspases (Xiang *et al*, 1996). In fibroblasts BCL-2 also appears to act prior to caspase activation and is able to delay the onset of apoptosis (McCarthy *et al*, 1997). Although BCL-2 effectively suppresses all DEX-induced loss of clonogenicity (Figure 6) we cannot rule out that we are also observing a delay which would be apparent at later time points. Transfection of BCL-2 has been reported to inhibit apoptotic features but not loss of clonogenicity in HeLa treated with aphidicolin (Yin and Schimke, 1995) or etoposide (Lock and Stribinskiene, 1996)

and similar results were seen with mouse B cells treated with etoposide (Kamesaki *et al*, 1993). Although these results suggest that BCL-2 is acting subsequent to loss of clonogenicity it has been shown that loss of clonogenicity after etoposide treatment is most likely caused by the inability of the cells to resolve DNA damage (Lock and Stribinskiene, 1996). Our findings that BCL-2 blocks the loss of clonogenicity induced by DEX (a non-genotoxic agent) are consistent with either BCL-2 or its regulators acting at or before the commitment point. The finding that DEX treated BCL-2 expressing cells still undergo DEX-induced cell shrinkage (R Benson *et al*. submitted) is in agreement with the finding that BCL-2 does not alter glucocorticoid transactivation but appears to alleviate repression of AP-1 activity (Miyashita *et al*, 1997) which has previously been shown to be required for DEX-induced apoptosis (Helmberg *et al*, 1995). Since DEX-induced commitment precedes, by several hours, the morphological and biochemical markers of apoptosis including loss of mitochondrial membrane potential our results are in agreement with previous cellular, genetic and *in vitro* studies placing BCL-2 at the onset of apoptotic signalling. (Cosulich *et al*, 1996; McCarthy *et al*, 1997; Shaham and Horvitz, 1996; Strasser *et al*, 1994; Susin *et al*, 1996).

The finding that BCL-2 is able to impede release of a zVAD-fmk sensitive protease (AIF) from mitochondria has led to the suggestion that AIF release represents a rate-limiting factor in cell death (Susin *et al*, 1996). However, our observations that loss of mitochondrial  $\Delta\Psi$  occurs after commitment to death (Figure 5) and that zVAD-fmk, which blocks AIF activity *in vitro* (Susin *et al*, 1996), does not block or delay clonogenicity (Figures 2B and 5B) suggest that DEX-induced clonogenic death does not require release of AIF. Recent observations have indicated that it is release of cytochrome c from mitochondria and not AIF or loss of mitochondrial  $\Delta\Psi$  which is both required for apoptosis and is blocked by BCL-2 expression (Kluck *et al*, 1997; Yang *et al*, 1997). Furthermore, cytochrome c release was shown to activate DEVD-specific caspases and preceded alterations in chromatin (Kluck *et al*, 1997; Yang *et al*, 1997) suggesting that it may be responsible for commitment to death. Although we show that changes in mitochondrial  $\Delta\Psi$  occur after commitment we can not rule out earlier cytochrome c release leading to the loss of clonogenicity observed.

The clonogenicity assay we have described is a direct measure of *functional* commitment to cell death that does not rely on known biochemical or morphological phenotypes associated with dying cells and, as such, it complements existing apoptotic assays. Like time-lapse video microscopy (McCarthy *et al*, 1997), the clonogenic assay described will register all dead cells at the point of sampling and any that die subsequently. However, although the fate of individual cells cannot be monitored with the precision of video-microscopy, assessment of commitment in the clonogenicity assay does not depend on visual cues such as cell shrinkage or membrane changes and can therefore determine commitment in the absence of morphological change. Since we have established cell culture conditions whereby *all* untreated cells are capable of generating a clone, an examination of the number of cells exhibiting known apoptotic features during treatment will establish whether

these features are likely regulators of commitment or simply appear during the later execution phase. For example, after 32 h of DEX treatment the percentage of cells which are committed to death, measured by clonogenicity, greatly exceeds the percentage of cells with condensed chromatin (Figure 1) ruling out chromatin condensation as a necessary component of commitment. Similar calculations have also shown that loss of mitochondrial  $\Delta\Psi$  must also occur after commitment to death (Figure 5). We have further illustrated the usefulness of this assay by examining whether inhibition of caspases or expression of BCL-2 either block or delay cell death. From kinetic and clonogenic analysis of cells treated with DEX and caspase inhibitors it is clear that, although visible death may be delayed, the total number of cells which eventually die, even when washed free of drug, remains unaffected. In contrast to the effect of caspase inhibitors, expression of BCL-2 although unable to alter DEX-induced cell shrinkage, blocks all signs of death including loss of clonogenicity.

## Materials and Methods

### Cell culture

A fresh cell line was derived from the parental CCRF CEM C7A stock (Norman and Thompson, 1977) by single cell cloning. CEM<sub>neo</sub> and CEM<sub>bcl-2</sub> cells, stably transfected with the retro-viral vectors pZipNeo and pZipBcl-2 respectively (Martin *et al*, 1995), were kindly provided by Dr. Seamus Martin (La Jolla Institute for Allergy and Immunology, La Jolla CA, USA). All cells were maintained in a 5% CO<sub>2</sub> humidified incubator at 37°C in Optimem 1 medium (Gibco BRL, Paisley, UK) supplemented with either 5% fetal bovine serum (FBS) for CEM C7A cells or 10% FBS for CEM<sub>neo</sub> and CEM<sub>bcl-2</sub> cells. Cells were treated with either 5  $\mu$ M DEX or solvent control as described previously (Wood *et al*, 1994). The protease inhibitor Z-VAL-ALA-ASP-CH<sub>2</sub>F (zVAD-fmk), (Enzyme System Products Inc., Dublin, CA, USA), was dissolved in DMSO and used at a final concentration of 50  $\mu$ M. Control cultures received solvent only.

### Clonogenic assay

The clonogenicity assay was based on a standard limiting dilution assay (Furth *et al*, 1981) and measures the ability of every cell in the culture to divide and form a clone. Briefly, diluted test cultures were plated out at a density of between 0.33 and 10 cells/well in round bottom 96-well plates. After two weeks incubation the number of wells showing clear growth (over 200 cells) were counted and clonogenicity determined by applying  $\%Clonogenicity = \{\ln(96/-ve\ wells)/plate\ density\} \times 100$  based on the Poisson distribution essentially as described by Furth *et al*, 1981). All dilutions used in the clonogenicity assay were sufficient to reduce the concentration of any DEX in the starting culture to non-toxic levels.

### Assessment of nuclear morphology

Apoptosis was measured in test cultures by assessment of nuclear morphology. Chromatin condensation and nuclear fragmentation were detected by acridine orange (5  $\mu$ M) staining and fluorescence microscopy as described previously (Wood *et al*, 1994). Images of control and treated cells were obtained by fluorescence microscopy of

cells washed in PBS, fixed with 2% formaldehyde in PBS and stained with 811  $\mu$ M Hoechst 33342. Images were recorded using a Photonic Science video camera and digitized using Improvision Openlab software. Flow cytometric analysis of chromatin condensation was achieved by washing test cultures in PBS and incubating with 4.5  $\mu$ M Hoechst 33342 for 1 h at 37°C to achieve steady state staining. Samples were analyzed using a Becton Dickinson FACS Vantage flow cytometer as described (Benson *et al*, 1996).

### Mitochondrial membrane potential

Mitochondrial membrane potential was measured using the J-aggregate forming dye, 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) essentially as described by Cossarizza *et al*. 1993). Briefly, cells between 2–8  $\times 10^5$  cells/ml were incubated for 10 min at 37°C with JC-1 at a final dye concentration of 10  $\mu$ g/ml. The cells were then washed and resuspended in PBS. Analysis was performed on a Becton Dickson FACS Vantage flow cytometer and the instrument was configured with a 530 nm (bandwidth 30) filter in front of FL1 and a 610 nm (bandwidth 20) in front of FL2. We found that a 610 nm filter was superior to the 585 nm (bandwidth 42) filter since it reduced cross channel breakthrough with minimal decrease in J-aggregate fluorescence signal. Electronic compensation of FL2-FL1 was set depending on the relative PMT settings of FL1, FL2, but in general, it varied from 20–50%. Fluorescence was read on a log scale and 2D contour plots of 610 *versus* 530 nm fluorescence generated. The monomer fluorescence was used as a guide to check for consistent loading of different samples.

### Immunoblotting

For each sample a lysate was prepared and 20  $\mu$ g of protein separated by SDS–PAGE (10% resolving gel, 4.5% stacking gel) and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-PVDF, Amersham Life Sciences, Aylesbury, UK). The blot was blocked with 5% dried milk in PBS and then incubated for 2 h at room temperature with the primary antibody, murine anti-PARP, (kindly donated by Drs. S. Aoufouchi and C. Milstein, MRC Centre, Cambridge UK). The immunoreactive proteins were visualised using rabbit horse radish peroxidase conjugated anti-mouse-IgG (1 : 1000, Sigma, Poole, UK) and enhanced chemiluminescence (ECL) (Amersham Life Sciences, Aylesbury, UK).

### Transmission electron microscopy

Transmission electron microscopy was performed on control and treated cultures. Cells were washed in 0.1 M sodium cacodylate buffer, pH 7.4, fixed in 2% glutaraldehyde/2% paraformaldehyde, stained with 2% osmium tetroxide and then incubated overnight in 0.5% uranyl acetate at 4°C. Cells were then dehydrated through an alcohol gradient before embedding in Spurr's resin (TAAB Laboratories, Reading, UK). Samples were sectioned into ultrathins (50 nm), stained with 2% uranyl acetate in 70% ethanol and 0.3% lead citrate in 0.1 M NaOH and examined using a Philips 400 transmission electron microscope.

### Assessment of DNA integrity by field inversion gel electrophoresis (FIGE)

Cells ( $1.5 \times 10^6$  sample) were collected, washed in nuclear buffer (10 mM Tris HCl pH 7.5, 150 mM NaCl, 5mM MgCl, 1 mM EDTA) and resuspended in nuclear buffer containing proteinase K (20  $\mu$ g/ml). Low

melting temperature agarose (1.5%) in nuclear buffer was added to the samples before pipetting into a flat bottom 96 well plate. Once set, the agarose plugs were incubated at 37°C, overnight, in lysis buffer (10 mM TrisHCl pH 8.8, 10 mM NaCl, 25 mM EDTA, 1% w/v Na laurylsarcosine) containing 200 µg/ml proteinase K. Plugs were washed three times for 1 h in TE and stored in 50 mM EDTA (pH 8) at 4°C. Plugs were loaded into a 1.5% agarose gel and wells sealed with 1.5% low melting temperature agarose in nuclear buffer. Electrophoresis was carried out as described (Oberhammer *et al*, 1993), after which the gel was stained with cyber green (1 : 10 000, Molecular Probes, Eugene, OR, USA) in running buffer (10 mM Tris-acetate, pH 8.6, 0.5 mM EDTA) for 20 min. DNA was visualised by UV illumination and photographed using an Appligene Imager. Molecular weight markers of 50–1000 kbp, purchased from Sigma, were used.

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