Cell Death and Differentiation (2006) 13, 607–618

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Functional dissociation of ΔΨm and cytochrome *c* release defines the contribution of mitochondria upstream of caspase activation during granzyme B-induced apoptosis

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Received 28.12.04; revised 15.7.05; accepted 05.8.05; published online 16.9.05
Edited by G Melino

Abstract

Loss of Bid confers clonogenic survival to granzyme B-treated cells, however the exact role of Bid-induced mitochondrial damage – upstream or downstream of caspases – remains controversial. Here we show that direct cleavage of Bid by granzyme B, but not caspases, was required for granzyme B-induced apoptosis. Release of cytochrome *c* and SMAC, but not AIF or endonuclease G, occurred in the absence of caspase activity and correlated with the onset of apoptosis and loss of clonogenic potential. Loss of mitochondrial trans-membrane potential (ΔΨm) was also caspase independent, however if caspase activity was blocked the mitochondria regenerated their ΔΨm. Loss of ΔΨm was not required for rapid granzyme B-induced apoptosis and regeneration of ΔΨm following cytochrome *c* release did not confer clonogenic survival. This functional dissociation of cytochrome *c* and SMAC release from loss of ΔΨm demonstrates the essential contribution of Bid upstream of caspase activation during granzyme B-induced apoptosis.

Cell Death and Differentiation (2006) **13,** 607–618.
doi:10.1038/sj.cdd.4401772; published online 16 September 2005

Keywords: granzyme B; cytochrome *c*; apoptosis; mitochondrial outer membrane permeabilization; mitochondrial transmembrane potential

Abbreviations: CL, cytotoxic lymphocyte; TRAIL, TNF-related apoptosis-inducing ligand; MOMP, mitochondrial outer membrane permeabilization; SMAC, second mitochondrial activator of caspases; AIF, apoptosis-inducing factor; EndoG, endonuclease G; ΔΨm, mitochondrial transmembrane potential; zVAD-fmk, *Z*-Val-Ala-Asp-CH_2F; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone

Introduction

Cytotoxic lymphocytes kill tumor cells or virus-infected cells by transferring granule enzymes (granzymes) into their target cell cytoplasm. In the target, granzymes activate specific cell death pathways by cleaving distinct cellular substrates.[1–4] Granzyme B has been shown to induce apoptosis, a physiological form of cell death that is orchestrated by caspases,[4–6] however, the mechanism of caspase activation during granzyme B-induced apoptosis, and specifically the role of mitochondria in this process, remains unclear.

Several lines of evidence put forward by multiple laboratories have shown that granzyme B induces early release of soluble proteins from the mitochondrial intermembrane space (MIS) via cleavage of the proapoptotic Bcl-2 family member Bid.[7–13] One of these mitochondrial proteins, cytochrome *c*, initiates an apoptosome complex that recruits and activates caspase-9.[14] Second mitochondrial activator of caspases (SMAC)/Diablo, which is also released from the MIS deregulates endogenous caspase inhibitors, the inhibitor of apoptosis proteins (IAPs) allowing unfettered caspase activation.[15,16] We have previously shown that granzyme B efficiently cleaves Bid after Asp75.[11,13] Further, loss of Bid or overexpression of Bcl-2 blocked granzyme B-induced release of cytochrome *c* from the MIS and the treated cells survived in long-term proliferation assays.[13,17,18] Inhibition of caspases blocked several features of apoptosis but did not block cytochrome *c* release or the eventual death of cells.[11,13,19] These data support a critical initiating role for Bid-induced MOMP upstream of caspases in granzyme B-induced apoptosis.

Mitochondrial transmembrane potential (ΔΨm), an electrical potential required for several mitochondrial functions including ATP generation, is dramatically reduced early during apoptosis. Loss of ΔΨm is therefore routinely used as an indicator of mitochondrial damage. Recently, the mitochondria of MCF7 cells were shown to maintain their ΔΨm following treatment with granzyme B. As MCF7 cells are deficient in caspase-3, it was concluded that caspase-3 activation was required upstream of mitochondrial participation in granzyme B-induced apoptosis. In this model, Bid cleavage was proposed to be a consequence of caspase-3 activation, possibly following direct cleavage of caspase-3 by granzyme B. In support of this, granzyme B has been shown to activate caspases directly in cell-free experiments[20–22] and caspases have been shown to cleave Bid at Asp59.[9] A critical

role for Bid-induced MOMP downstream of caspase activation has previously been reported during death receptor-induced apoptosis. Indeed, Bid-/- cells or cells that are designated 'type II' are resistant to death receptor-induced apoptosis, even though caspase-8 activation occurs upstream of Bid.[23,24] In these cells, low level of caspase-8 activation is amplified via Bid-mediated mitochondrial outer membrane permeabilization (MOMP).

The disparate conclusions from these two sets of studies, one set that predict caspases are required for granzyme B-induced mitochondrial damage and another that mitochondrial damage is required for activation of caspases, are based on results obtained by exclusively measuring either cytochrome *c* release or loss of $\Delta\psi$m as an indicator of mitochondrial damage. We have therefore closely assayed the release of several soluble proteins released from the MIS and loss of $\Delta\Psi$m during granzyme B-induced apoptosis. We found that cytochrome *c* and SMAC were released independently of caspase activity, however, active caspases were required for the release of apoptosis-inducing factor (AIF) and endonuclease G (EndoG). We also observed caspase-independent loss of $\Delta\Psi$m at a similar time to cytochrome *c* release however $\Delta\Psi$m was rapidly regenerated in the absence of caspase activity. Loss of $\Delta\psi$m was not required for rapid granzyme B-induced cell death and sustained maintenance of $\Delta\psi$m did not convey clonogenic survival to granzyme B-treated cells. This clear functional dissociation of loss of $\Delta\psi$m and cytochrome *c* release supports the critical role of MOMP upstream of caspase activation during granzyme B-induced apoptosis. Importantly, these data are consistent with inducers of apoptosis that activate Bcl-2-regulated, mitochondria-dependent pathways to apoptosis.

Results

Direct cleavage of Bid at Asp75 is required for granzyme B-induced apoptosis

Granzyme B induces apoptosis in HeLa cells, as evidenced by rounding, shrinkage, blebbing and annexin V binding before plasma membrane rupture (Figure 1a) Further, cell shrinkage (reduced forward scatter as detected by flow cytometry) and annexin V binding were blocked by the caspase inhibitor zVAD-fmk (Figure 1b) or overexpression of Bcl-2.[11]

Bcl-2-regulated cell survival or cell death is believed to be determined by the ratio of pro- or antiapoptotic Bcl-2 family members. To determine whether Bid is cleaved by granzyme B or caspases during granzyme B-induced apoptosis, we expressed Bid with a mutated granzyme B cleavage site (BidD75E) or a mutated caspase cleavage site (BidD59E) in HeLa cells expressing Bcl-2 (Figure 2a). HeLa-Bcl-2 cells were resistant to granzyme B-induced apoptosis, however, coexpression of BidD59E (mutated caspase cleavage site) resensitized the HeLa-Bcl-2 cells to granzyme B-induced apoptosis (Figure 2b). In contrast, HeLa-Bcl-2 cells expressing BidD75E (mutated granzyme B cleavage site) remained resistant to granzyme B-induced apoptosis despite the presence of an intact caspase cleavage site in Bid (Figure 2b). To test that the expressed BidD75E was functionally active, we treated the HeLa-Bcl-2 cells expressing the mutant Bid proteins with TRAIL, which acts via caspase-8-mediated cleavage of Bid. HeLa-Bcl-2 cells were resistant to TRAIL-induced apoptosis, however coexpression of BidD75E (mutated granzyme B cleavage site), but not BidD59E (caspase cleavage site) resensitized HeLa-Bcl-2 cells to TRAIL-induced apoptosis (Figure 2c). Importantly, HeLa-Bcl-2 cells and HeLa-Bcl-2 cells expressing BidD59E or BidD75E showed similar expression of Bcl-2 as determined by immunocytochemistry (Figure 2a) and these cells were all resistant to actinomycin D, a cytotoxic drug that does not require Bid to induce apoptosis (Figure 2d). These findings provide compelling evidence that granzyme B-mediated cleavage of Bid at D75 is required to initiate cell death, and that Bid cleavage is not secondary to caspase activation by granzyme B.

Granzyme B-induced MOMP is caspase independent but caspase-3 is required for sustained loss of $\Delta\Psi$m

As discussed, exclusive analysis of either cytochrome *c* release or loss of $\Delta\Psi$m during granzyme B-induced apoptosis has resulted in disparate conclusions about the role of mitochondria upstream[11,13] or downstream of caspases.[28] To investigate the consequences of Bid cleavage during granzyme B-induced apoptosis, we followed the release of several soluble proteins from the MIS and loss of $\Delta\Psi$m. We found that cytochrome *c* and SMAC were released into the cytoplasm of cells treated with granzyme/perforin B both in the presence or absence of zVAD-fmk (Figure 3a). AIF and EndoG were also released into the cytoplasm of cells treated with granzyme B/perforin, however, release of these proteins was blocked by zVAD-fmk (Figure 3a). These data show that SMAC and cytochrome *c* release occur upstream of caspase activation while AIF and EndoG are released downstream of caspase activation during granzyme B-induced apoptosis. These data are consistent with recent studies that showed Bid induces the release of cytochrome *c* and SMAC but not AIF or EndoG from isolated mitochondria[32,33] and that release of AIF and EndoG were caspase dependent during mitochondria-dependent apoptosis induced by cytotoxic drugs.[32]

We also found that caspase-3 was cleaved to its active p17 subunit in HeLa cells following treatment with granzyme B/perforin but was only cleaved to a p20 subunit in the presence of zVAD-fmk or in HeLa cells over-expressing Bcl-2 (Supplementary Figure 1). This is consistent with our previous studies in Jurkat cells, which predicted that granzyme B has the ability to cleave caspase-3 to its p20 subunit[12,21] but requires the release of mitochondrial proteins in addition to autolytic cleavage to allow full activation.[12]

Using cytochrome *c* as an indicator of caspase-independent MOMP, we compared MOMP and loss of $\Delta\Psi$m in HeLa cells treated with granzyme B/perforin in the presence or absence of zVAD-fmk. We found that virtually all HeLa cells treated with granzyme B/perforin, became annexin V positive (Figure 3bi), released cytochrome *c* from their mitochondria (Figure 3bii) and lost $\Delta\Psi$m (Figure 3biii). In the presence of zVAD-fmk the granzyme B-treated cells remained annexin V

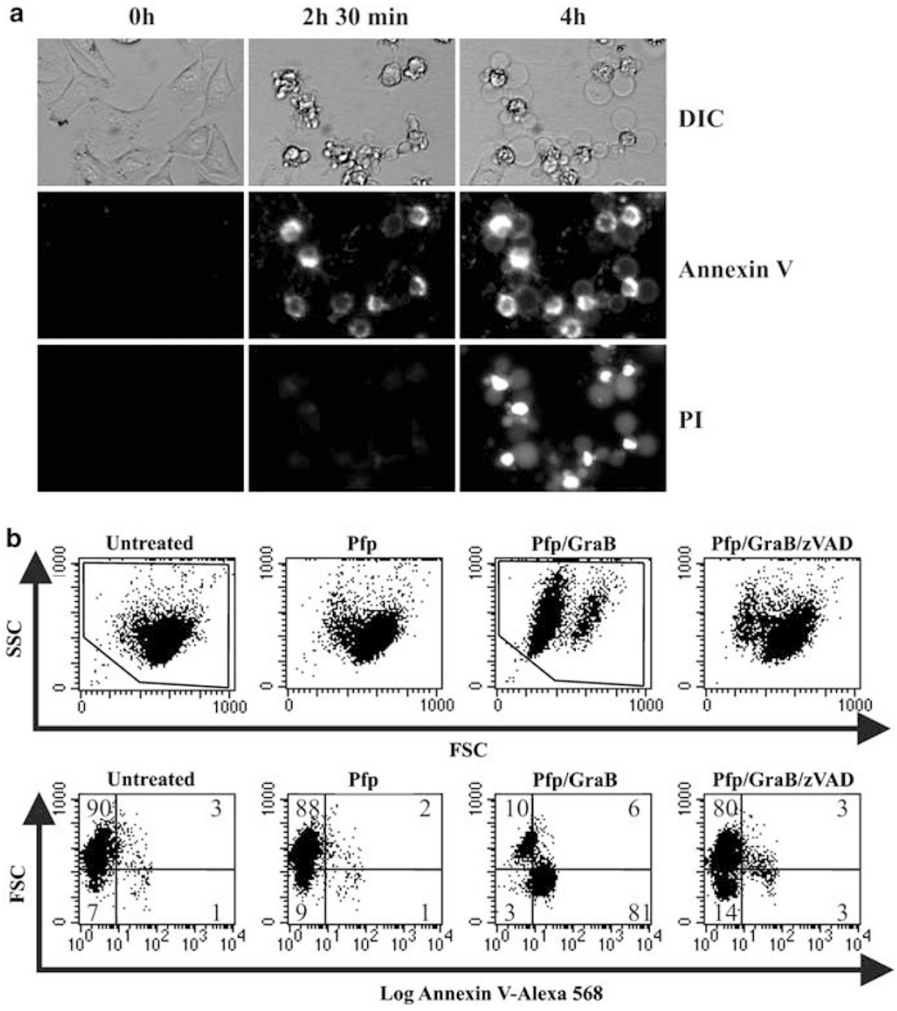

Figure 1 Granzyme B induces apoptosis in HeLa cells. (**a**) HeLa cells were treated with granzyme B (25 nM) and perforin (1 nM) and incubated at 37°C in media containing Annexin V-FITC and PI (50 ng/ml). Images of morphology (DIC), annexin V binding and PI uptake were taken after 2.5 and 4 h. Rounding and annexin V binding was observed in all cells before PI uptake. Images are representative of three independent experiments. (**b**) HeLa cells were treated with granzyme B (25 nM) and/or perforin (1 nM) and/or zVAD-fmk (100 μM) and incubated at 37°C in media for 4 h. Cells were stained with Annexin V-alexa568 and analysed by flow cytometry. Annexin V-alexa568 was detected in FL-3. FACS plots of forward scatter [(FSC indicative of cell size) *versus* side scatter ((SSC) indicative of granularity) and FSC *versus* annexin V-568 are presented and are indicative of 10 independent experiments. Numbers indicate the percentage of cells in each quadrant. The polygon in the FSC *versus* SSC plot is indicative of the gating strategy used throughout this manuscript

negative (Figure 3bi) but the mitochondria of these cells still released cytochrome *c* (Figure 3bii). Of note, analysis by flow cytometry and Western blot showed that a small amount of cytochrome *c* was retained in the mitochondria of cells treated with granzyme B/perforin in the presence of zVAD-fmk, however the log scale used in the FACS analysis shows that this accounts for less than 10% of total cytochrome *c*. Although the mitochondria of these cells had released the majority of cytochrome *c*, we found that the mitochondria maintained their $\Delta\Psi$m (Figure 3biii). The ability of mitochondria to maintain $\Delta\Psi$m following cytochrome *c* release in the absence of caspase activity was observed regardless of whether recombinant (Figure 3b) or native granzyme B (Supplementary Figure 2) was used, or if its delivery was by perforin (Figure 3), pneumolysin (Supplementary Figure 2) or adenovirus (Figure 4).

Consistent with the data shown in Figure 3b, a recent study found that MCF7 cells that lacked caspase-3 maintained their $\Delta\Psi$m during granzyme B-induced apoptosis.[28] Maintenance of $\Delta\Psi$m in these cells was interpreted to show that caspases were required for mitochondrial involvement during granzyme B-induced apoptosis. We wished to determine whether granzyme B could induce cytochrome *c* release in these cells independent of caspase-3 activity. We found that MCF7 cells treated with granzyme B/perforin remained annexin V negative (Figure 3ci) and maintained their $\Delta\Psi$m (Figure 3ciii) following granzyme B treatment, however the mitochondria of these cells released cytochrome *c* (Figure 3cii). MCF7 cells in which caspase-3 expression was reconstituted were sensitive to granzyme B-induced apoptosis as measured by annexin V staining, released cytochrome *c* from their mitochondria and the mitochondria of these cells lost their $\Delta\Psi$m (not shown).

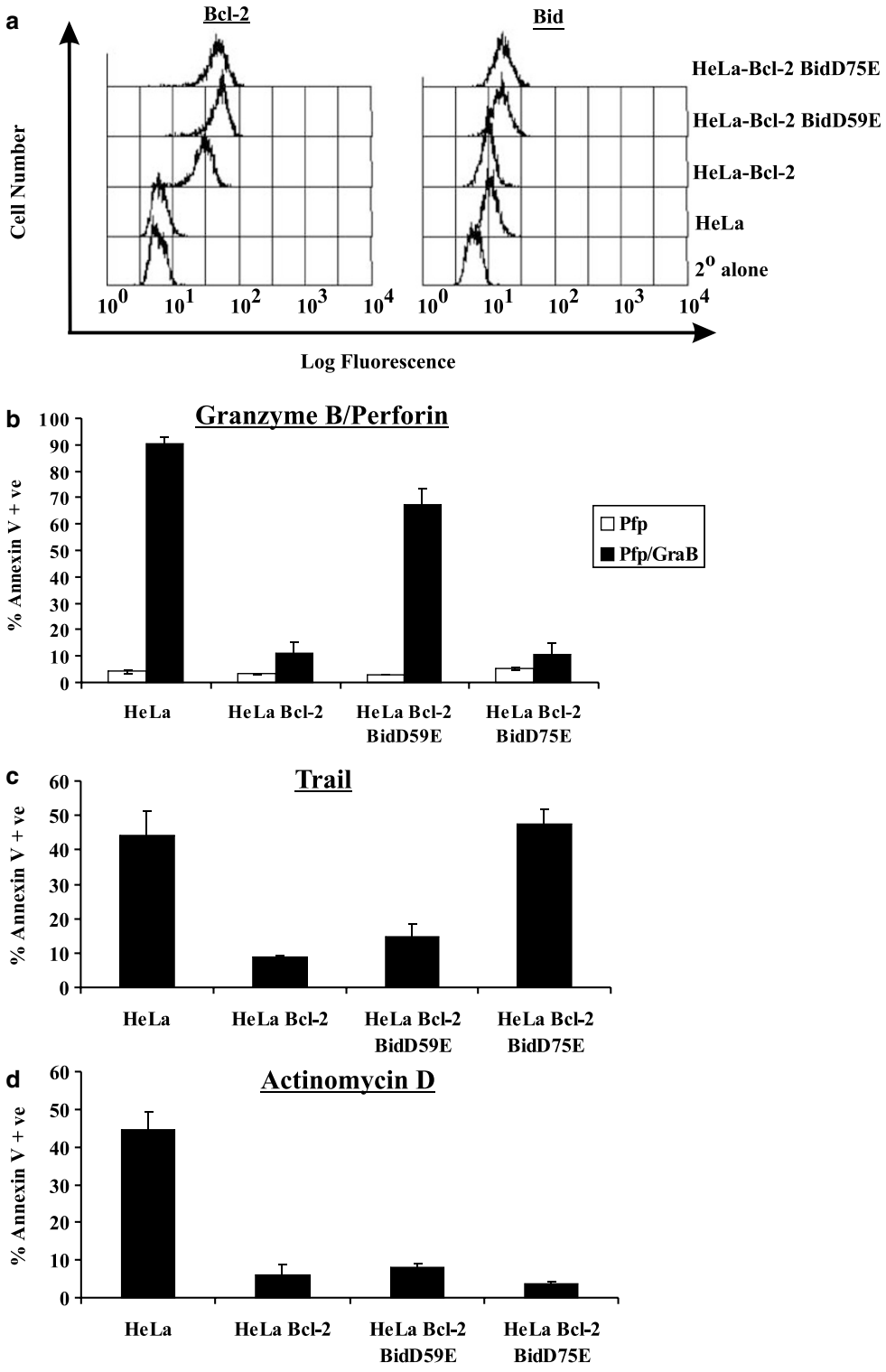

Figure 2 Bid cleavage by granzyme B specifically at Asp75 is required for efficient cell death. (**a**) HeLa cells, HeLa-Bcl-2 and HeLa-Bcl-2 cells overexpressing mutated Bid (D59E or D75E) were analysed by immunocytochemistry. Expression of Bid and Bcl-2 was evident by an increase in fluorescence intensity. (**b**) HeLa cells, HeLa-Bcl-2 and HeLa-Bcl-2 cells overexpressing mutated Bid (D59E or D75E) were treated with perforin (1 nM) and granzyme B (25 nM). Cells were harvested after 4 h and assayed for annexin V binding by flow cytometry. Data are presented as the mean $\pm$ S.D. of triplicate samples, representative of four independent experiments. (**c**) HeLa cells, HeLa-Bcl-2 and HeLa-Bcl-2 cells overexpressing mutated Bid (D59E or D75E) were treated with TRAIL (10 ng/ml) and assayed for annexin V binding after 4 h. Data are presented as the mean $\pm$ S.D. of triplicate samples, representative of three independent experiments. (**d**) HeLa cells, Hela cells overexpressing Bcl-2 (HeLa-Bcl-2) and Hela-Bcl-2 cells overexpressing mutated Bid (D59E or D75E) were treated with actinomycin D (1 μM) and assayed for annexin V binding after 24 h

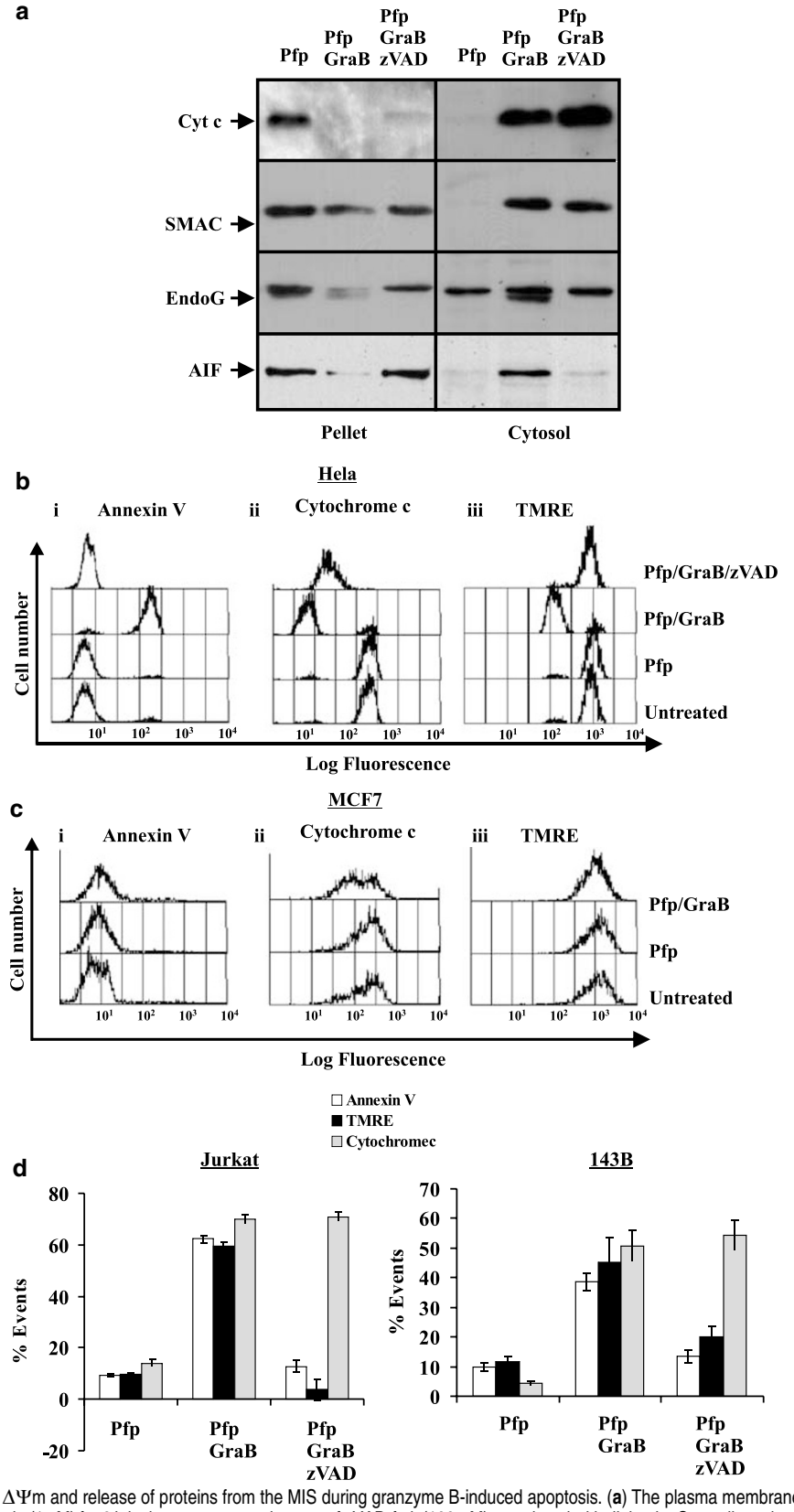

Figure 3 Sustained loss of $\Delta\Psi$m and release of proteins from the MIS during granzyme B-induced apoptosis. (**a**) The plasma membranes of HeLa cells treated with granzyme B (25 nM) and perforin (1 nM) for 2 h in the presence or absence of zVAD-fmk (100 μM) were lysed with digitonin. Cytosolic and pellet fractions were harvested and subjected to Western blot analysis for Cytochrome *c*, SMAC, AIF and EndoG. HeLa cells (**b**) MCF7 cells (**c**) Jurkat cells and 143 B cells (**d**) treated with granzyme B (25 nM) and perforin (1 nM) were stained with Annexin V, anticytochrome *c*/anti-mouse-PE or TMRE as indicated and analysed by flow cytometry. Data is presented as the mean $\pm$ S.D. of triplicate samples, representative of three independent experiments

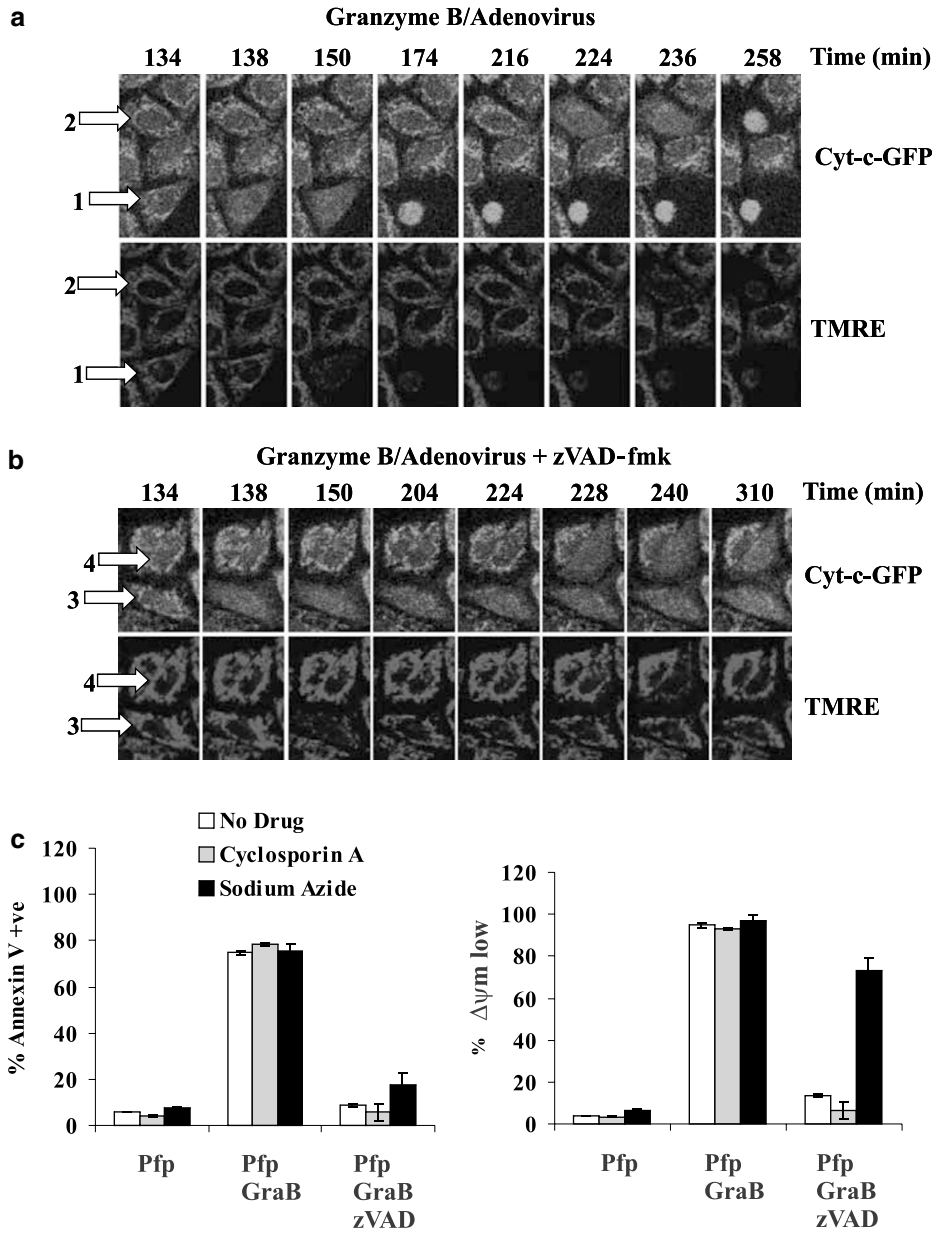

Figure 4 $\Delta\Psi$m is lost following cytochrome *c* release during granzyme B-mediated apoptosis but is regenerated by the electron transport chain in the absence of caspase activity. HeLa cells expressing cytochrome *c*-GFP and stained with TMRE were treated with granzyme B (25 nM) and adenovirus (10 PFU) in (**a**) the absence or (**b**) the presence of zVAD-fmk (100 μM) and followed by time-lapse confocal microscopy. (**a**) Two cells depicted 1 and 2 (top panel) undergo apoptosis. Cell #1 undergoes cytochrome *c* release by 138 min but $\Delta\Psi$m is not lost until 150 min. The cell then rounds up by 174 min. Cell #2 follows a similar time course with cytochrome *c* release evident by 224 min. In the presence of zVAD-fmk (**b**), cell #3 releases cytochrome *c* by 138 min. $\Delta\Psi$m is almost completely lost by 150 min, but is regenerated by 204 min. Cell #4 follows a similar time course with cytochrome *c* release evident by 228 min. (A full time line can be seen in supplementary movies.) (**c**) HeLa cells treated with treated with granzyme B (25 nM) and perforin (1 nM) in the presence or absence of zVAD-fmk and/or cyclosporin A (40 μM) or sodium azide (2 mM) were assayed for annexin V binding or TMRE. Data are presented as the mean $\pm$ S.D. of triplicate samples, representative of three independent experiments

Similar to what was observed in HeLa cells, we also found that loss of $\Delta\Psi$m was caspase dependent while cytochrome *c* release was caspase independent in Jurkat cells and 143B osteosarcoma cells treated with granzyme B/perforin (Figure 3d). Our data show that mitochondrial damage occurs both upstream (release of SMAC and cytochrome *c*) and downstream (release of AIF and EndoG and sustained loss of $\Delta\Psi$m) of caspase activation during granzyme B-induced apoptosis.

Changes in $\Delta\Psi$m during granzyme B-induced apoptosis occur independent of permeability transition

It is known that during apoptosis induced by several stimuli, mitochondria lose their $\Delta\Psi$m following cytochrome *c* release, however, $\Delta\Psi$m can be regenerated if caspase activity is blocked.[34–36] To investigate whether $\Delta\Psi$m regeneration can occur during granzyme B-mediated apoptosis, we followed

HeLa cells expressing cytochrome *c*-GFP treated with granzyme B and adenovirus, using time-lapse confocal microscopy. We observed that $\Delta\Psi$m was lost following cytochrome *c*-GFP release during apoptosis (Figure 4a and supplementary Figure 3), however, when caspase activity was blocked with zVAD-fmk, the loss of $\Delta\Psi$m was only transient (Figure 4b and supplementary Figure 4). As shown by the four representative cells that demonstrate these features (Figure 4), a distinct and specific chronology of apoptotic events was observed. In the absence of caspase inhibition (Figure 4a), cytochrome *c* became cytoplasmic in cell #1 between 134 and 138 min. $\Delta\Psi$m was virtually depleted over the following 12 min, then the cell became progressively rounded over the following 30 min. Similarly, in cell #2 cytochrome *c* release was observed between 216 and 224 min. $\Delta\Psi$m was depleted over the following 12 min, and the cell became round within the following half hour. In the presence of zVAD-fmk (Figure 4b), a different sequence of events was observed. As exemplified in cell #3 cytochrome *c*-GFP became cytoplasmic by 138 min, $\Delta\Psi$m was depleted within the following 12 min, however, the cell maintained a flat morphology and the mitochondria regenerated their $\Delta\Psi$m over the following hour. In the final example (cell #4), cytochrome *c* release was observed by 228 min, followed by loss of $\Delta\Psi$m by 240 min and regeneration of $\Delta\Psi$m over the following hour. These data clearly demonstrate that cytochrome *c* release occurs before loss of $\Delta\Psi$m during granzyme B-mediated apoptosis, that caspases are not required for cytochrome *c* release, however, caspases are required for the sustained loss of $\Delta\Psi$m. These data definitively show that granzyme B-induced cytochrome *c* release is independent of caspase activity, that loss of $\Delta\Psi$m occurs after cytochrome *c* release and that active caspase-3 is required for sustained loss of $\Delta\Psi$m.

Rapid loss and regeneration of $\Delta\Psi$m is a characteristic of permeability transition.[37] During permeability transition, mitochondria lose their transmembrane potential and swell until the outer membrane ruptures releasing the contents of the MIS into the cytoplasm (as reviewed in Waterhouse *et al*.[38]). Conversely, cytochrome *c* participates in generation of mitochondrial trans-membrane potential by transferring electrons from complex III to IV of the electron transport chain. Loss of cytochrome *c* may therefore impact on loss and regeneration of $\Delta\Psi$m. To investigate whether loss and regeneration of $\Delta\Psi$m during granzyme B-induced apoptosis was a consequence of cytochrome *c* release or permeability transition, we treated cells with granzyme B/perforin in the presence of the permeability transition inhibitor, cyclosporin A or the complex IV inhibitor sodium azide (to prevent the contribution of cytochrome *c* to the electron transport chain). Neither cyclosporin A nor sodium azide blocked granzyme B-induced apoptosis or loss of $\Delta\Psi$m (Figure 4c) in the absence of the caspase inhibitor zVAD-fmk. Interestingly, in the presence of zVAD-fmk, sodium azide but not cyclosporin A prevented the regeneration of $\Delta\Psi$m following cytochrome *c* release. These data show that neither permeability transition nor complex IV activity are required for granzyme B-induced cell death, however, complex IV activity was required for the regeneration of $\Delta\Psi$m following cytochrome *c* release. These data are consistent with our previous studies, which showed that the concentration of cytochrome *c* maintained within the cytoplasm of HeLa cells treated with cytotoxic drugs is sufficient to drive electron transport, but only if caspase activity is blocked.[36]

Loss of $\Delta\Psi$m does not contribute to rapid death of granzyme B-treated cells

Caspase-3 targets the mitochondria to ensure sustained loss of $\Delta\Psi$m, however, the consequence of sustained loss of $\Delta\Psi$m for apoptosis is not clear. ATP levels have been proposed to act as a molecular switch between apoptosis and necrosis.[39] Apoptosis occurs only if there is sufficient ATP to support this active process, however if ATP is limiting, the cell deviates to necrotic death. It therefore remains possible that sustained loss of $\Delta\Psi$m might contribute to rapid death of cells in the absence of caspase activation. To investigate whether sustained loss of $\Delta\Psi$m can contribute to rapid granzyme B-induced cell death, we treated HeLa cells with granzyme B/perforin in the presence or absence of zVAD-fmk and/or carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), an protonophore that depolarizes mitochondria (Figure 5). In this assay, the mitochondria would not be able to regenerate $\Delta\Psi$m following MOMP. We found that zVAD-fmk inhibited annexin V binding (as a measure of apoptosis) and release of ^{51}Cr (a measure of plasma membrane permeabilization) regardless of whether FCCP was present or not (Figure 5a and b), indicating that loss of $\Delta\Psi$m did not contribute significantly to cell death in the absence of activated caspases. Importantly, FCCP did not block perforin/granzyme B-mediated apoptosis, nor was it toxic to cells when applied alone (Figure 5a and b). These data support the idea that while MOMP plays a critical role in rapid apoptotic cell death induced by granzyme B, loss of $\Delta\Psi$m *per se* is not a key functional event during to granzyme B-induced cell death.

MOMP but not loss of $\Delta\Psi$m correlates with loss of clonogenic potential

In healthy respiring cells, $\Delta\Psi$m is required for the production of ATP by oxidative phosphorylation therefore regeneration of $\Delta\Psi$m may prevent death of granzyme B-treated cells. To investigate the importance of $\Delta\Psi$m in maintaining cell survival, we treated HeLa and HeLa-Bcl-2 cells with granzyme B and perforin in the presence or absence of zVAD-fmk (Figure 6). Consistent with previous studies, we found that HeLa cells treated with granzyme B/perforin became annexin V positive (Figure 6a), released cytochrome *c* (Figure 6b) and had reduced clonogenic survival (Figure 6c). Similar cells treated with zVAD-fmk, in which cytochrome *c* was released but had regenerated $\Delta\psi$m, also had reduced clonogenic survival (Figure 6c). In contrast, the number of colonies obtained from Bcl-2 overexpressing cells treated with granzyme B, in which cytochrome *c* had remained in the mitochondria (Figure 6b), was similar to that of cells treated with perforin alone (Figure 6c).

Loss and regeneration of $\Delta\Psi$m is likely to cause cell stress that could contribute to loss of clonogenic potential following cytochrome *c* release. To investigate this we mimicked loss

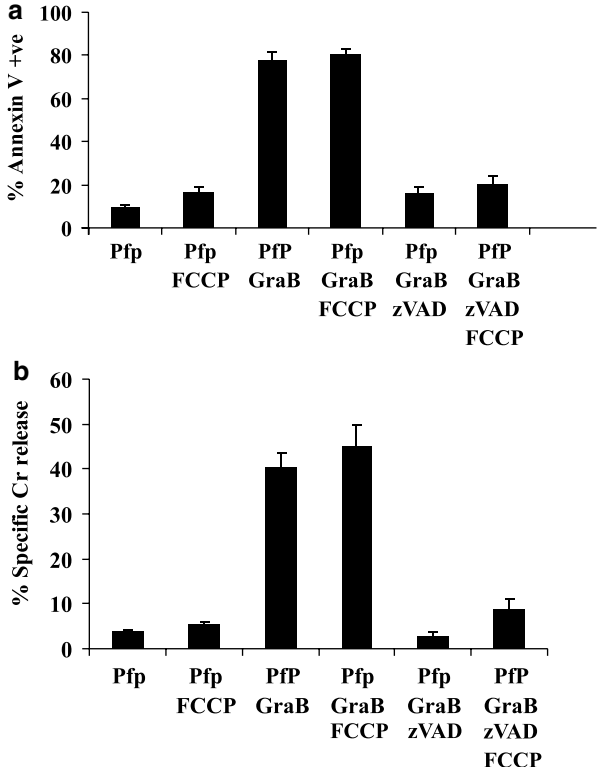

Figure 5 Loss of $\Delta\Psi$m does not promote to rapid granzyme B-induced cell death. HeLa cells treated with granzyme B (25 nM) and perforin (1 nM) for 4 h in the presence or absence of zVAD-fmk (100 μM) or FCCP (10 μM) were assayed for (**a**) annexin V staining or (**b**) release of preloaded ^{51}Cr. Data are presented as the mean $\pm$ S.D. of triplicate samples, representative of three independent experiments

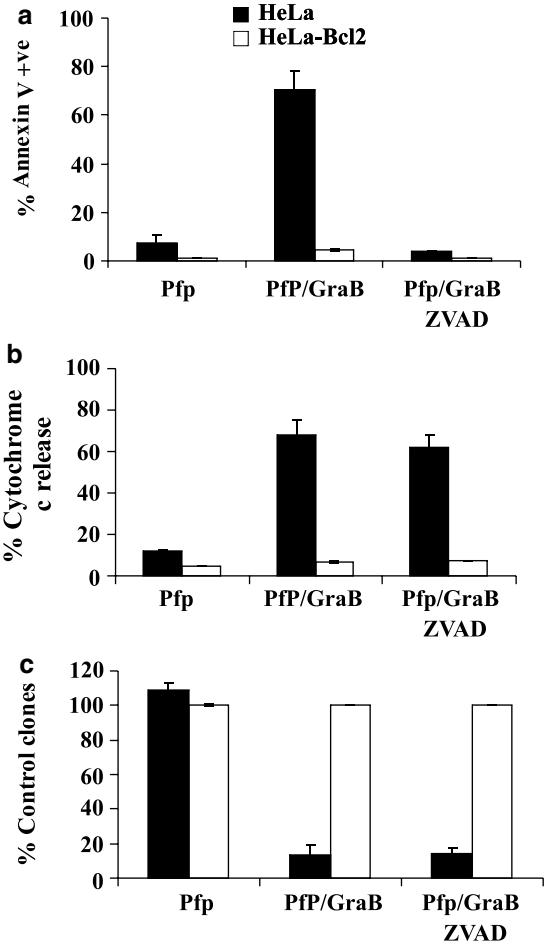

Figure 6 MOMP correlates with loss of clonogenic potential. HeLa or HeLa-Bcl2 cells treated with granzyme B (25 nM), perforin (1 nM) and or zVAD-fmk (100 μM) were incubated for 1 h and washed. Annexin V binding (**a**) and cytochrome *c* release (**b**) was measured at 4 h. (**c**) In all, 2×10^4 cells from each sample were seeded by serial dilution in 24 well plates. Colonies were counted after 7 days and expressed as a percentage of control. Data are presented as the mean $\pm$ S.D. of triplicate samples, representative of three independent experiments

and regeneration of $\Delta\Psi$m by treating cells with FCCP for 4 h followed by washing to remove the FCCP. Aliquots of cells were stained with TMRE at 1 and 4 h to confirm FCCP-induced loss of $\Delta\Psi$m in more than 99% of treated cells (not shown). We found that if FCCP was not removed, approximately 50% cell death was evident after 72 h (Figure 7a) and no colonies were evident after 7 days (Figure 7b). In samples where cells were treated with FCCP for 4 h and the FCCP was removed by washing in media (to mimic loss and regeneration of $\Delta\Psi$m), the percentage of death after 3 days was similar to that of untreated cells as was the number of colonies present after 7 days. These data show that loss and regeneration of $\Delta\Psi$m following cytochrome *c* release is not sufficient to cause loss of clonogenic potential. Further, our data show that cytochrome *c* release, but not loss of $\Delta\Psi$m, is an accurate indicator for clonogenic survival of granzyme B-treated cells.

Discussion

It is well documented that granzyme B induces caspase-dependent apoptosis, however, the mechanism by which granzyme B activates caspases, specifically the role of mitochondria in this process, is contentious. Several laboratories have shown that granzyme B can directly cleave and activate the proapoptotic Bcl-2 family member Bid,[7,8,10–13] which facilitates caspase activation by releasing proteins from the MIS. Recent studies have challenged this hypothesis and propose that mitochondrial disruption occurs secondarily to caspase activation.[28,40] In this study, we have presented data that demonstrates a clear initiating role for MOMP in granzyme B-induced cell death and resolves the apparent discrepancies between these studies.

We have shown that granzyme B cleaves Bid directly at D75 and that Bid cleavage is not secondary to caspase activation by granzyme B. Further, we have shown that release of cytochrome *c* and SMAC occurs upstream of caspase activation during granzyme B-induced cell death. Caspases then secondarily target the mitochondria to induce the release of AIF and EndoG and ensure the sustained loss of $\Delta\Psi$m (Figure 8). These data show that AIF and EndoG do not play an initiating role in caspase activation during granzyme B-induced apoptosis and are unlikely to play a role in

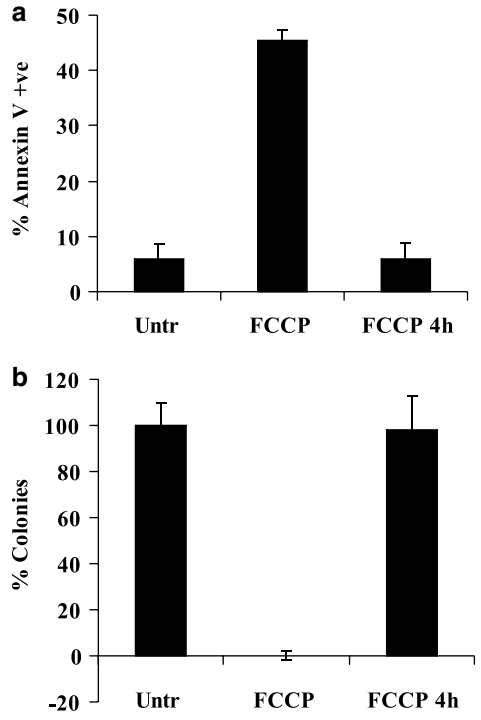

Figure 7 Transient loss of $\Delta\Psi$m in the absence of cytochrome *c* release does not commit a cell to death. HeLa cells were treated with FCCP (10 μM). An aliquot of treated cells was washed in media after 4 h. In all, 2×10^4 cells from each sample were seeded by serial dilution in 24-well plates. FCCP (10 μM) was present in the media used for seeding the FCCP-treated cells. (**a**) The percentage of death was analysed by annexin V binding after 72 h. (**b**) Colonies were counted after 7 days and expressed as a percentage of control

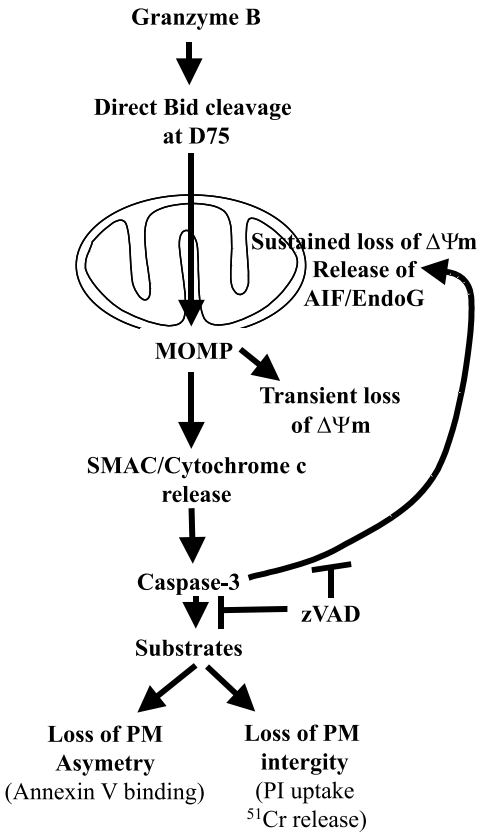

Figure 8 Pathways to granzyme B-induced cell death. Granzyme B directly cleaves and activates Bid to induce caspase-independent mitochondrial outer membrane permeabilization. This results in the SMAC release, cytochrome *c* release and a transient loss of $\Delta\psi$m. Cytochrome *c* then activates caspase-3 which feeds back on the mitochondria ensuring sustained loss of $\Delta\psi$m and release of AIF and EndoG. Caspase-3 also orchestrates the ordered dismantling of the cell by apoptosis

caspase-independent cell death. These data are consistent with recent studies, which showed that Bid induces the release of cytochrome *c* and SMAC but not AIF or EndoG from isolated mitochondria[32,33] and that AIF and EndoG release was caspase dependent during drug-induced apoptosis.[32] These data reaffirm the critical role of Bid in the regulation of granzyme B-mediated cell death upstream of caspase activation.

We found that mitochondria of HeLa cells, Jurkat cells or 143B cells treated with zVAD-fmk and MCF7 cells (that lack caspase-3) underwent caspase-independent MOMP as evidenced by cytochrome *c* release, however, the mitochondria maintained their $\Delta\Psi$m in the long term after a brief initial loss. The data presented here indicate that caspase-3 is not required for MOMP. Furthermore, caspase-3 is not required for the transient loss of $\Delta\Psi$m occurring immediately following MOMP, but is necessary for the permanent loss of $\Delta\Psi$m. It has recently been shown that caspase-dependent cleavage of p75 in complex I of the electron transport chain is required for sustained loss of $\Delta\Psi$m following cytochrome *c* release during drug-induced apoptosis.[34] Our data suggest that cleavage of p75 by caspase-3 is also required to ensure sustained loss of $\Delta\Psi$m during granzyme B-induced apoptosis.

It has recently been shown that Bid itself does not induce loss of $\Delta\Psi$m,[32] however, we clearly see loss and regeneration of $\Delta\Psi$m during granzyme B-induced apoptosis. This is consistent with the observation that Bid itself does not induce rapid loss of $\Delta\Psi$m in isolated mitochondria, because cell-free systems do not account for the cellular demand on ATP.[36] Further, we have shown that the concentration of cytochrome *c* used in cell-free systems does not necessarily reflect the concentration of cytochrome *c* maintained within intact cells.[36]

It has also been reported that granzyme B can induce mitochondrial depolarization in the absence of cytochrome *c* release, Bid, Bax or Bak.[40] Our data in four different cell lines shows that at concentrations of granzyme B that efficiently induce apoptosis, active caspases were required to induce sustained loss of $\Delta\Psi$m. This suggests that granzyme B itself does not directly induce caspase-independent loss of $\Delta\Psi$m (Figure 3). In a previous report,[17] we showed that the concentrations of granzyme B used by Thomas *et al.*,[40] were 100 times higher than that required to induce apoptosis in various primary and transformed cell lines. It therefore remains possible that at high concentrations, granzyme B can induce caspase-independent loss of $\Delta\Psi$m. The physiological role for granzyme B at these concentrations remains unclear. We showed that regeneration of $\Delta\Psi$m requires active respiration. It is therefore possible that some cells with a high dependence of oxidative phosphorylation may not easily recover $\Delta\Psi$m. Regardless, we would predict that any cell that

can regenerate $\Delta\Psi$m following drug-induced apoptosis, should be capable of regenerating their $\Delta\Psi$m following treatment with granzyme B/perforin.

Maintenance of $\Delta\Psi$m reflects critical bioenergetic processes, therefore loss of this potential might be thought to impact on cellular viability. Indeed zVAD-fmk has been shown to be ineffective in blocking rapid granzyme B-induced death in some cells. Preventing regeneration of $\Delta\psi$m with FCCP did not lead to rapid caspase-independent cell death in HeLa cells. This suggests that sustained loss of $\Delta\psi$m is not the reason that zVAD-fmk significantly delays death in some cells but does not block death in others. These results, combined with the fact that Bcl-2 (which blocks MOMP) but not zVAD (which maintains $\Delta\psi$m but not MOMP) maintains the clonogenic potential of granzyme B-treated cells,[11,13,18,41] further supports the hypothesis that MOMP, rather than loss of $\Delta\psi$m (although the latter may be a result of MOMP) is the critical event in granzyme B-induced cell death.

Granzyme B has been shown to activate caspase-3 directly in cell-free systems. Further, we have shown that granzyme B can cleave caspase-3 to a p20 form in the presence of zVAD-fmk or in Bcl-2 overexpressing cells (Supplementary Figure 1). It could therefore be argued that the mitochondrial pathway to caspase activation is only required where autoactivation of caspase-3 is held in check by IAPs. While there is no direct evidence for or against this argument, the fact that primary Bid−/− cells isolated from various tissues were relatively resistant to granzyme B-induced apoptosis,[17] suggests that mitochondrial involvement in caspase activation is required in normal physiological settings. We cannot however preclude that in some cells in which IAPs are low, that granzyme B can directly activate caspases. It is also possible that in cases where MOMP is blocked, for example if Bid is absent or Bcl-2 is overexpressed, that granzyme B can directly activate caspases if delivered in sufficient quantities.

These data clearly demonstrate that cytochrome *c* release is an accurate indicator of mitochondrial damage upstream of caspases during granzyme B-induced apoptosis, however $\Delta\psi$m is not. Indeed studies that predict that mitochondria are only involved downstream of caspase-3 based on failure to drop $\Delta\psi$m have failed to account for the involvement of cytochrome *c* release upstream of caspase-3 activity. These data also demonstrate a clear difference between death receptor-induced apoptosis, in which initiator caspases may be required upstream of Bid in certain cell types, and granzyme B-induced apoptosis in which Bid cleavage occurs upstream of any requirement for initiator caspase activity.

Materials and Methods

Materials

^{51}Cr (as sodium dichromate) was from Amersham Biosciences (UK). *Z*-Val-Ala-Asp-CH_2F (zVAD)-fmk was from Enzyme Systems Products (Dublin, CA, USA). Annexin V-FLUOS was from Roche (Indianapolis, IN, USA). Annexin V-alexa568 and tetramethylrhodamine ethylester (TMRE) were from Molecular Probes (Eugene, OR, USA). TUNEL reagents were from Boehringer Mannheim (Sydney, Australia). Cyclosporin A was from Novartis (Basel, Switzerland). Mouse anti-cytochrome *c* antibody (Clone 6HB12 for immunocytochemistry and 7H8.2C12 for Western blotting) and anticaspase-3 were from BD Pharmingen (San Diego, CA, USA). Anti-SMAC and anti-EndoG was from ProSci (San Diego, CA, USA), anti-AIF clone E1 was from Santa Cruz (Santa Cruz, CA, USA). PE-labelled anti-mouse Ig was from Silenus, Australia and anti-EE was from Berkley antibody company (Berkley, CA, USA), Human-leucine-zipper-(TNF-related apoptosis-inducing ligand) TRAIL was a kind gift from Immunex corporation (Seattle, WA, USA). Native and recombinant CTL granule proteins were isolated according to established methods. Perforin was purified essentially according to Liu *et al*.,[25] Granzyme B was purified as described by Trapani *et al*.,[26] or Sun *et al*.,[27] The granzyme B was free of granzyme A (BLT esterase) activity and perforin. Cell culture reagents were from Gibco-BRL (Melbourne, Australia). All other chemicals were from Sigma (St. Louis, IL, USA).

Cell culture

All cells used in this study were cultured at 37°C in a humidified CO_2 incubator in medium supplemented with 2 mM glutamine and 10% fetal bovine serum. HeLa cells expressing cytochrome *c*-GFP (referred to in this manuscript as HeLa), HeLa cells expressing Bcl-2 (HeLa-Bcl-2) were cultured in DMEM. HeLa-Bcl-2 cell were transfected with constructs containing EE-tagged-Bid in which Glu was substituted for Asp at position 59 (D59E) or position 75 (D59E) using Fugene (Roche). Cells expressing Bid were selected in DMEM containing hygromycin (100 μg/ml). 143B cells were maintained in DMEM, MCF7 human breast cancer cells and Jurkat cells were cultured in RPMI-1640.[28]

Flow cytometry (Annexin V binding, loss of $\Delta\Psi$m, cytochrome c release)

Annexin V binding:[29] Following exposure to granzyme B and perforin, cells were incubated for 5 min at 37°C in 100 μl of annexin V buffer (10 mM Hepes NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) containing 0.5 μl annexin V-FLUOS or annexin V-Alexa568. Annexin V FLUOS fluorescence was detected by flow cytometry in FL-1, annexin V-alexa568 was detected in FL-3.

For $\Delta\Psi$m: cells were incubated for 20 min at 37°C in media containing 50 nM TMRE. TMRE fluorescence was detected by flow cytometry as described.[30]

For cytochrome *c* release;[31] cells were incubated for 5 min on ice in 100 μl of digitonin buffer (80 mM KCl, 50 ng/ml digitonin and 1 mM EDTA in PBS). An aliquot was assayed to ensure at least 95% of cells were Trypan blue positive. The permeabilized cells were fixed in 4% paraformaldehyde in PBS, washed in PBS and incubated for 1 h in blocking buffer (3% BSA, 0.05% Saponin in PBS). Cells were incubated at least overnight at 4°C with anticytochrome *c* diluted 1 : 200 in blocking buffer, washed in PBS and incubated at room temperature for at least 1 h in PE-labelled secondary antibody. The cells were washed in PBS and PE fluorescence was detected by flow cytometry in FL-2.

Immunocytochemistry

Cells were fixed in paraformaldehyde (4%) at 4°C for 10 min. Cells were incubated in blocking solution (50 : 50 v/v NH_4Cl in PBS (50 mM) and foetal calf serum (FCS)) for 1 h and washed in permeabilization buffer (saponin (0.2%), FCS (1%), sodium azide (0.02%) in PBS). Cells were incubated at 4°C for 30 min in permeabilization buffer containing anti-Bcl-2 (10 : 1 hybridoma supernatant) or anti-EE (1 : 500). Cells were washed and incubated at 4°C for 30 min in permeabilization buffer containing

anti-mouse IgG-FITC (1 : 200) and analysed by flow cytometry. FITC was detected in FL-1.

Western blotting

Cells treated with granzyme B (25 mM)/perforin (1 nM) were harvested and incubated at 4°C for 5 min with ice-cold cell lysis and mitochondria intact (CLAMI) buffer (120 mM KCl, 1 mM EDTA, 50 μg/ml digitonin in PBS). Cells were pelleted by centrifugation ($400 \times g$ for 5 min). Supernatants (cytosol) were removed and pellets (mitochondria) were lysed in universal immunoprecipitation buffer (UIB (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EDTA and complete protease inhibitors)). Lysates were resolved by SDS-PAGE and transferred to PVDF (Immobilon P). Blots were blocked using 5% skim milk powder in PBS containing tween (0.05%). Cytochrome *c*, SMAC, AIF and EndoG were detected using the specific primary antibodies diluted 1 : 1000 in blocking buffer followed by species-specific secondary antibodies conjugated to horseradish peroxidase. Horseradish peroxidase was detected by enhanced chemi-luminescence-PLUS (Pierce). Each membrane was analysed for several proteins by reprobing.

Specific ^{51}Cr release

In all, 1×10^6 cells were incubated in 100 μl media with ^{51}Cr (75 μCi) for 1 h at 37°C. Cells were washed three times in Hank's balanced salt solution containing 0.3% BSA and 20 mM Hepes to remove the unincorporated ^{51}Cr and resuspended at 2×10^5 cells/ml. Cells were incubated with granzyme B/perforin for 4 h at 37°C and the supernatant harvested using a SKATRON supernatant collection system (SKATRON, Norway). ^{51}Cr released (cpm) into the supernatant was detected using a Wallac Wizard 1470 automatic gamma counter (Perkin Elmer, Boston, MA, USA). In each case the spontaneous release of radiolabel over the time of the assay was no higher than 10% of the total incorporated radioactivity.

Microscopy

Images of morphology were obtained using an Olympus IX81 microscope. Cells were treated with granzyme B (25 nM) and perforin (1 nM). Differential interference contrast, and fluorescence images were taken of the same field of cells using an XY controlled microcope stage (PRIOR) controlled by MetaMorph software. Images were processed using MetaMorph software. For time-lapse microscopy, cells were plated overnight in 35 mm culture dishes in DMEM. The media was replaced with phenol red free, serum free, DMEM and the dishes were maintained at 37°C on a temperature-controlled microscope stage (Harvard Apparatus Inc., Natic MA, USA) before adding granzyme B (25 nM) and adenovirus (10 PFU). Cytochrome *c*-GFP translocation and TMRE fluorescence were followed by time-lapse confocal microscopy using a Nikon Eclipse TE 300 microscope and a Bio-Rad MRC 1024 confocal. Cytochrome *c*-GFP and TMRE were excited by a 488 nm laser line attenuated 96%. GFP was detected in PMT2 and TMRE was detected in PMT1. Images were taken every 2 min. Images were processed using Image J software (NIH).

Acknowledgements

NJW is a Peter Doherty Fellow and JAT a Senior Principal Research Fellow of the National Health and Medical Research Council, Australia. RWJ is a Wellcome Trust Senior International Research Fellow. This study was supported by Program Grants funding to JAT, RWJ and PIB from NHMRC. We thank AMRAD Australia Ltd, for support to NJW. Bid constructs were from Dr. D Huang, Walter and Eliza Hall Institute, Melbourne Australia. PLO was from Dr. J Paton, Childrens and Womens Hospital, Adelaide, Australia. Replication-deficient adenovirus was provided by Chris Bleackley, University of Alberta, Canada. MCF7 cells and MCF7 expressing caspase-3 were provided by Chris Froelich, Evanston Northwestern Healthcare Research Institute, MD. We thank Sarah Ellis for assistance with microscopy and Ruth Kluck for sharing resources and critical analysis of the manuscript. We thank members of the Cancer Immunology Program for technical help and valuable discussions.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)