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Oxidative stress induces caspase-independent retinal apoptosis *in vitro*

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Abstract

Apoptosis is the mode of cell death in retinitis pigmentosa (RP), a heterogeneous group of retinal degenerations. The activation of the caspase proteases forms a pivotal step in the initiation and execution phase of apoptosis in many cells. Inhibition of caspases has been reported to prevent apoptosis in many model systems. However, we demonstrate the absence of caspase activation during retinal cell apoptosis *in vitro* which involves phosphatidylserine (PS) externalisation, DNA nicking and cell shrinkage. In addition, zVAD-fmk, DEVD-CHO and BD-fmk, inhibitors of the caspases, were unable to alter the characteristics or kinetics of apoptosis, implying that retinal cell death *in vitro* follows a caspase-independent pathway. We have previously demonstrated the ability of reactive oxygen species (ROS) to act as mediators of retinal cell apoptosis *in vitro* as well as the ability of antioxidants to prevent retinal cell apoptosis. Here we demonstrate the oxidative inactivation of caspases in this model of retinal apoptosis and provide evidence for an oxidative stress driven cell death pathway that does not involve caspase activity and which retains key features of apoptotic cell death. Furthermore, our data indicates that apoptotic events such as PS exposure, DNA nicking and cell shrinkage may occur independently of caspase activity. *Cell Death and Differentiation* (2000) **7,** 282–291.

Keywords: apoptosis; retina; retinitis pigmentosa; caspase-independent; reactive oxygen species

Abbreviations: BD-fmk, t-butyloxycarbonyl-aspartate-fluoromethylketone; DAPI, 4′-6 diamindino-2-phenylindole dichloride; DCFH/DA, dichlorofluorescein diacetate; DEVD-CHO, aspartate-glutamate-valine-aspartate-aldehyde; DEVD-*p*NA, aspartate-glutamate-valine-aspartate-*p*-nitroanilide; DTT, Dithiothreitol; GSH, Glutathione; JC-1, 5,5′,6-6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazole-carbocyanine iodide; PDTC, pyrrolidinedithiocarbamate; PS, phosphatidylserine; ROS, reactive oxygen species; RP, retinitis pigmentosa; TUNEL, terminal dUTP nick end labelling; z-VAD-fmk, benzyloxycarbonyl-valine-alanine-aspartate-fluoromethylketone

Introduction

Apoptosis is a form of programmed cell death which enables a cell to direct its own destruction. This form of cell death appears to be crucial for mammalian development and subsequent tissue homeostasis. Inappropriate apoptosis has been implicated in several neurodegenerative diseases such as Alzheimer's,[1] amyotrophic lateral sclerosis[2] and Huntington's,[1] as well as ischaemic damage[3] and several forms of retinal degeneration collectively referred to as retinitis pigmentosa (RP).[4–6] RP is characterised by an initial loss of night vision followed by a progressive loss of peripheral and central visual acuity. Histologically, degeneration of rod photoreceptors is associated with night blindness while degeneration of cone photoreceptors correlates with loss of peripheral and central visual fields. The molecular genetics underlying the pathology of RP is highly heterogeneous and at least 15 genes have been implicated in the aetiology of RP to date (Http://www.sph.uth.tmc.edu/retnet). Several of the implicated genes are involved in the rod phototransduction cascade[7] while others have been ascribed a role in retinoid cycling in the retina.[8,9] The exact mechanisms by which such genetic lesions result in the degenerative phenotype of RP is presently unclear however it is becoming evident that such diverse genetic defects converge on a common pathway for photoreceptor apoptosis.[4–6] At present, apoptotic photoreceptor cell death appears to represent the only unifying feature of all forms of RP, with the implication that components of the apoptotic machinery may serve as potential therapeutic targets applicable to all forms of RP.

Genetic studies of the nematode *C. elegans* have identified two genes, *ced-3* and *ced-4*, which are required for normal developmental cell death.[10] A third gene, *ced-9*, was found to be a negative regulator of apoptosis and to act antagonistically to *ced-3* and *ced-4* to prevent cell death.[10] Mammalian homologues of each of these genes have now been identified and are represented by the *bcl-2* gene family (*ced-9*),[11] Apaf-1 (*ced-4*)[12] and the caspase gene family (*ced-3*).[13] Caspase-1 was the first mammalian Ced-3 homologue identified[13] in what is now an expanding family of cysteine proteases of which caspase-3 displays the highest homology with the Ced-3 prototype.[14] The caspases are believed to function in the effector stage of apoptosis following an initiating signal.[15] Such signals may derive from the direct activation of death receptors such as Fas[16] or from the release of cytochrome-c from mitochondria.[17] Activation of caspases by cytosolic cytochrome-c requires the formation of an 'apoptosome' complex between cytochrome-c, Apaf-1

and procaspase-9 in an ATP dependent manner. Both release of cytochrome-c and the activation of procaspase-9 by Apaf-1 are inhibitable by Bcl-xL.[18,19] Once activated caspase-9 may initiate a cascade of caspase activation which ultimately leads to apoptosis.[20]

Many targets of caspase activity have now been identified and give strong support for the role of caspases as effectors of the apoptotic programme.[21] Caspase substrates include proteins which play a critical role in DNA replication,[22,23] DNA repair[24] cell survival signalling,[25] as well as proteins which regulate cytoskeletal reorganisation and cellular disassembly.[26,27] A caspase activated DNase (CAD) has also been identified; cleavage of its endogenous inhibitor (ICAD) by caspases allows its translocation to the nucleus where it catalyses the internucleosomal cleavage of chromosomal DNA.[28] Much of the consequences of caspase activity have been determined using a range of synthetic and viral inhibitors. Such inhibitors have been demonstrated to inhibit apoptosis in a range of cell types using a variety of inducing stimuli.[29] However, several studies have reported the inability of caspase inhibitors to prevent cell death in a number of model systems.[30–36] These studies suggest that cells which have committed to caspase activation are destined to die and that while the inhibition of caspases may prevent certain morphological and biochemical features of apoptosis their ultimate fate is cell death.

In this study, we report the apoptosis of retinal cells *in vitro* via a pathway that does not involve caspase activation and can not be prevented by caspase inhibitors. We show that the generation of reactive oxygen species and oxidative stress during retinal cell death results in the oxidative inactivation of the caspases. The cell death that follows features phosphatidylserine (PS) exposure on the outer surface of the plasma membrane, DNA nicking and cell shrinkage, indicating that these apoptotic events occur independently from caspase activation.

Results

Phosphatidylserine exposure, DNA nicking and cell shrinkage in primary retinal cell culture. Absence of low molecular weight DNA fragmentation

The spontaneous apoptosis of primary retinal cells cultured under serum free conditions has previously been described with respect to DNA nicking, nuclear condensation and cell shrinkage[37,38] (Figure 1B), all of which are established, characteristic, features of apoptosis. Approximately 75% of all cells in the mouse retina are photoreceptors, and 97% of these are rods. The remaining cells include retinal ganglion, bipolar, horizontal and amacrine cells. In this study we have analyzed other events typical of apoptotic retinal cell death such as exposure of PS on the outer leaf of the plasma membrane and low molecular weight, internucleosomal DNA fragmentation. Our data indicates that PS flipover is a feature of retinal cell death *in vitro* as measured by annexin-V-FITC binding (Figure 1A). However, low molecular weight, internucleosomal DNA fragmentation was not detected in retinal cell cultures using DNA agarose gel electrophoresis (Figure 1C), despite the labelling of these cells using the terminal dUTP nick end labelling (TUNEL) assay which fluorescently labels 3′ OH nicked ends in DNA (Figure 1D). This data indicates the presence of DNA strand nicks or possibly high molecular weight (300–500 kb) DNA degradation, as shown by the TUNEL assay, in the absence of low molecular weight fragmentation, as demonstrated by DNA gel electrophoresis, during *in vitro* retinal cell apoptosis. Analysis of nuclear morphology of retinal cells after 12 h in culture, using DAPI stained cells under a fluorescence microscope, demonstrates nuclear condensation and fragmentation as these cells undergo apoptosis (Figure 1E).

Caspase inhibitors zVAD-fmk, DEVD-CHO and BD-fmk fail to prevent retinal cell apoptosis

Caspase inhibitors have been widely reported to inhibit apoptosis in a large number of cell types induced to undergo apoptosis by a diverse range of stimuli. The apparent central role played by the caspases for the completion of the death program demonstrated in such studies prompted us to investigate their role in retinal cell apoptosis *in vitro*. Three peptide inhibitors of caspases were employed. DEVD-CHO has been reported to inhibit caspase-3, -7, -1 and -4,[26] while the zVAD-fmk peptide has a broader spectrum of inhibition, effective against all caspases tested to date, including caspases-3, -6, -8, -1, -2 and -4. The truncated zVAD-fmk analogue BD-fmk is a general cysteine protease inhibitor and has a still wider spectrum of inhibition against the caspases. The concentrations of Z-VAD-fmk, DEVD-CHO and BD-fmk used were 100, 100 and 80 μM respectively. The data shown in Figure 2A demonstrates the failure of all three caspase inhibitors to prevent retinal cell apoptosis as assessed by annexin-V binding. Jurkat cells treated with an apoptosis inducing anti Fas IgM in the presence or absence of the caspase inhibitors z-VAD-fmk, DEVD-CHO and BD-fmk, were used as a control to demonstrate the efficacy of these inhibitors to prevent apoptosis at the concentrations employed (Figure 2B). In addition to this, the caspase inhibitors used did not alter the kinetics of the death process or any other features of apoptosis as described in Figure 1 (Figure 2A,C,D, and data not shown).

Caspase-3 is not activated during retinal cell apoptosis

The failure of effective levels of caspase inhibitors to prevent apoptosis in retinal cultures encouraged us to investigate the activation status of the caspases during cell death. The activation of caspase-3 requires its proteolytic processing from the 32 kD pro-enzyme to the 17 kD active enzyme. Analysis of the levels of the 32 and 17 kD caspase-3 species using Western blot demonstrates the absence of active, 17 kD caspase-3 in cell lysates taken from retinal cultures undergoing apoptosis (Figure 3). The murine haematopoeitic 32D cell line was employed to demonstrate the ability to detect the 17 kD caspase-3 form, which was detectable following ultra violet irradiation of these cells (Figure 3). These data suggest that the failure of caspase inhibitors to prevent

retinal apoptosis is due to the non-participation of caspases in the death program of these cells. In addition to this, the levels of procaspase-3 present in retinal cells is significantly lower than that found in the murine haematopoietic 32D cell line (Figure 3). The differences in caspase-3 levels observed between these two cell types may be a reflection of the differences in the natural propensity of a dividing, short-lived haematopoietic cell and a post-mitotic, irreplaceable neuronal cell to activate the apoptotic death program.[39]

Caspases are oxidatively inactivated during retinal cell death

We have previously demonstrated a crucial role for reactive oxygen species (ROS) as mediators of retinal cell apoptosis *in vitro*.[38] This, together with studies demonstrating the sensitivity of caspases to redox state alterations,[40] suggested that the inactivation of caspase-3 during retinal cell apoptosis may be due to oxidative modification. Figure 4A demonstrates the ability of peroxide to inactivate the caspase activity in a cytosolic extract from anti-Fas IgM treated Jurkat cells. Jurkat cells were treated with apoptosis inducing anti-Fas IgM antibody and total cellular protein extracted. Jurkat cell lysates were then treated with increasing concentrations of hydrogen peroxide (50 to 800 mM) for 30 min at 4°C. Subsequent analysis of caspase activity as described in Materials and Methods demonstrated the inhibition of caspase (DEVDase) activity with increasing concentrations of hydrogen peroxide. Fifty mM hydrogen peroxide reduced caspase activity in anti-Fas treated Jurkat cell lysates by

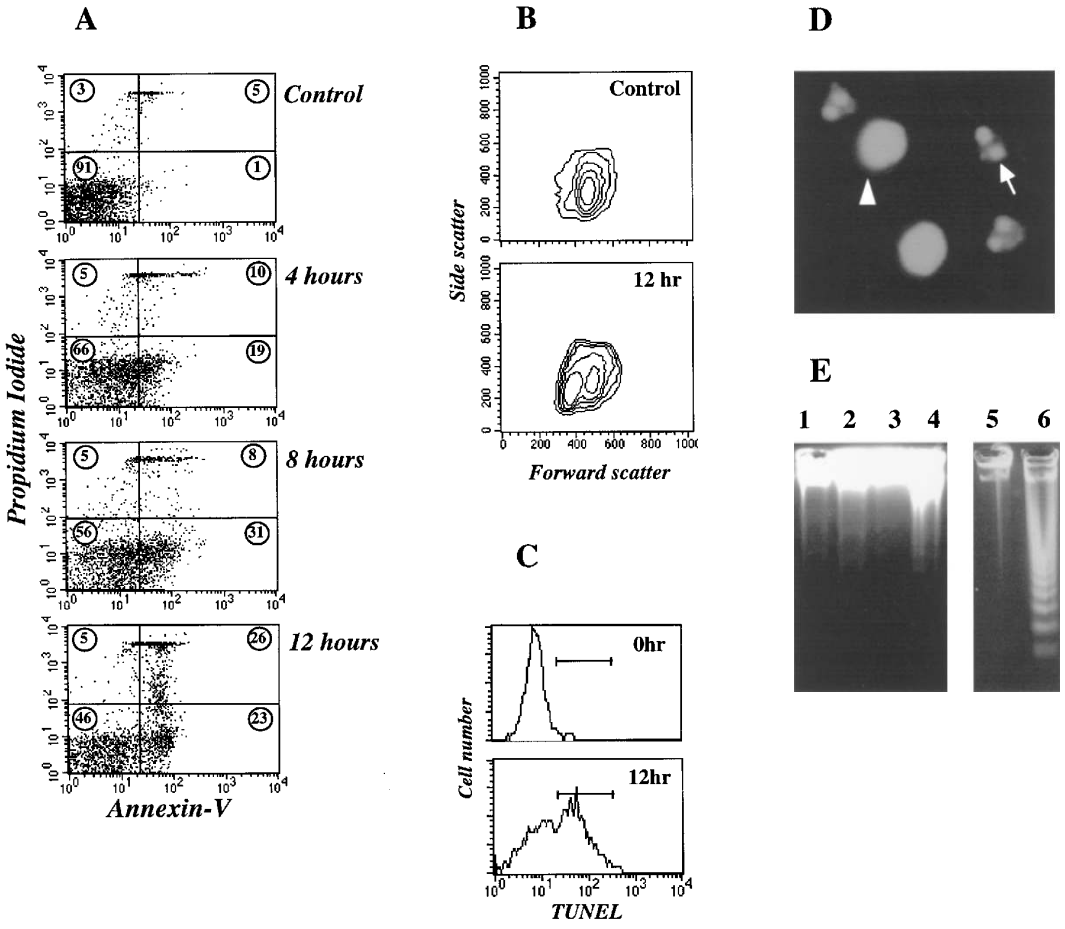

Figure 1 Phosphatidylserine (PS) exposure, cell shrinkage, and DNA nicking occur in the absence of low molecular weight, internucleosomal, DNA fragmentation during retinal cell death *in vitro*. (**A**) Retinal cell cultures show increased annexin-V-FITC binding over time (0 to 12 h) due to exposure of PS on the outer leaflet of the plasma membrane. This increase in annexin-v binding is followed by increased membrane permeability to propidium iodide (PI) due to secondary necrosis. (**B**) Retinal cells also display cell shrinkage over 12 h in culture as determined by a decrease in the forward and side light scattering properties of these cells. Forward and side scatter may be used as a measure of cell size and granularity respectively. (**C**) Low molecular weight, internucleosomal DNA fragmentation was not detected following 1.5% agarose electrophoresis of retinal cell extracts at 0, 4, 8 and 12 h (lanes 1, 2, 3, 4 respectively). Untreated and anti-Fas IgM treated (300 ng/ml for 4 h) Jurkat cell extracts (lanes 5 and 6 respectively) analyzed in the same way serve as negative and positive controls for DNA fragmentation respectively. (**D**) DNA nicking in retinal cells measured immediately after isolation and following 12 h in culture using the TUNEL assay which allows the fluorescent labelling of 3′OH ends on DNA. (**E**) DAPI staining of retinal cells 12 h after introduction into culture demonstrates the appearance of nuclear apoptotic morphology. Normal cell displays a diffuse nuclear staining with DAPI (arrowhead), while apoptotic nuclei are condensed and fragmented (arrow). The appearance of cells with apoptotic nuclear morphology coincided with the detection of TUNEL labelled and shrunken cells by flow cytometry

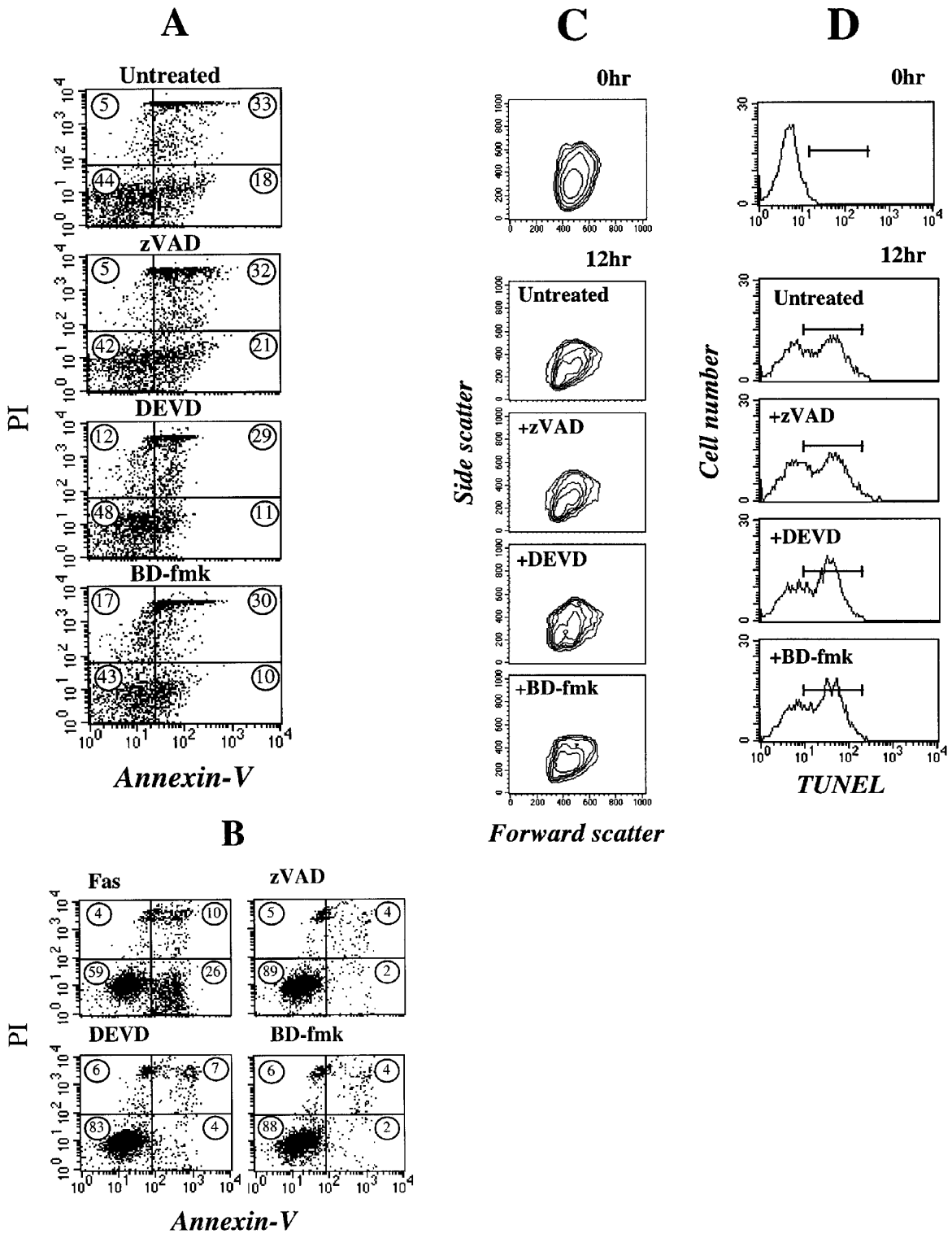

Figure 2 The caspase inhibitors zVAD-fmk, DEVD-CHO and BD-fmk do not prevent retinal cell death *in vitro*. (**A**) Treatment of retinal cell cultures with the caspase inhibitors does not prevent PS exposure. Retinal cells were incubated in the presence and absence of zVAD-fmk (100 μM), DEVD-CHO (100 μM) and BD-fmk (80 μM) and PS exposure measured by annexin-V binding after 12 h in culture. (**B**) zVAD-fmk (100 μM), DEVD-CHO (100 μM) and BD-fmk (80 μM) prevent PS exposure and apoptosis in anti-Fas IgM (300 ng/ml for 4 h) treated Jurkat cells. (**C**) zVAD-fmk (100 μM), DEVD-CHO (100 μM) and BD-fmk (80 μM) do not prevent retinal cell shrinkage measured after 12 h in culture. (**D**) DNA nicking in retinal cell cultures (measured after 12 h in culture), as determined by TUNEL, is not prevented by zVAD-fmk (100 μM), DEVD-CHO (100 μM) and BD-fmk (80 μM)

approximately 55% while 100 mM decreased caspase activity by approximately 75% (Figure 4A). It has been suggested that a critical cysteine group in the active site of the caspases makes them sensitive to thiol oxidation.[41] In order to investigate the means of caspase inactivation in cell lysates from Fas treated Jurkat cells incubated with peroxide, we determined the ability of the thiol reducing agent dithiothreitol (DTT) to restore caspase activity. As Figure 4B demonstrates, incubation of hydrogen peroxide (200 mM) treated lysates from anti-Fas treated Jurkat cells with 100 and 200 mM DTT restores caspase activity. This provides further evidence for the ability of ROS to inactivate the caspases through thiol oxidation. To determine whether oxidative inactivation of the caspases was responsible for their non-participation in retinal cell apoptosis we repeated this experiment using lysates recovered from retinal cells as they underwent apoptosis *in vitro*. Figure 4C shows that 100 to 400 mM DTT is capable of restoring caspase activity in retinal cell lysates in a manner similar to that seen in peroxide treated Jurkat lysates. While the concentrations of both hydrogen peroxide and DTT used in these experiments is quite high it is likely that lower concentrations would have yielded similar results if the incubation times were extended. These data suggest that the lack of caspase activation observed in retinal cells as they undergo apoptosis in culture is due to the oxidative inactivation of the caspases, most likely at the thiol group in the active site of these enzymes.

Reactive oxygen species (ROS) generation is an early event and precedes mitochondrial membrane potential disruption in retinal cell apoptosis

The involvement of ROS in photoreceptor apoptosis *in vitro* has previously been established[38] (and Table 1). As Figure 5B shows, the generation of intracellular ROS is an early event in retinal cell apoptosis *in vitro* with increased peroxide levels detectable as early as 2 h following introduction into culture. The loss of mitochondrial membrane potential has

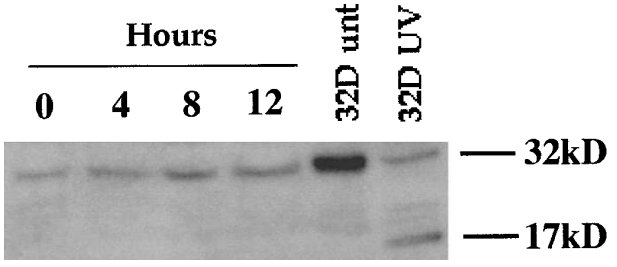

Figure 3 Immunoblot analysis of caspase-3 demonstrates absence of caspase-3 activation as retinal cell cultures undergo cell death. Equivalent quantities of total protein from retinal cell lysates taken at 0, 4, 8 and 12 h, and untreated and UV treated 32D cells were resolved using SDS–PAGE and transferred to a nitrocellulose membrane. The presence of pro-caspase-3 (32 kD) and the proteolytically active 17 kD species were determined by immunoblot analysis using anti-caspase-3 antibody. Protein extracts from retinal cultures were isolated following 4, 8, and 12 h of culture, as well as freshly isolated retinal cells (0 h). While the 32 kD pro-caspase species is present at all time points analyzed, the active 17 kD species is absent up to 12 h. 32D cells demonstrate the processing of pro-caspase-3 (32 kD) to the active 17 kD fragment as these cells undergo apoptosis. 32D protein lysates extracted 16 h after a 10 min exposure to ultraviolet irradiation

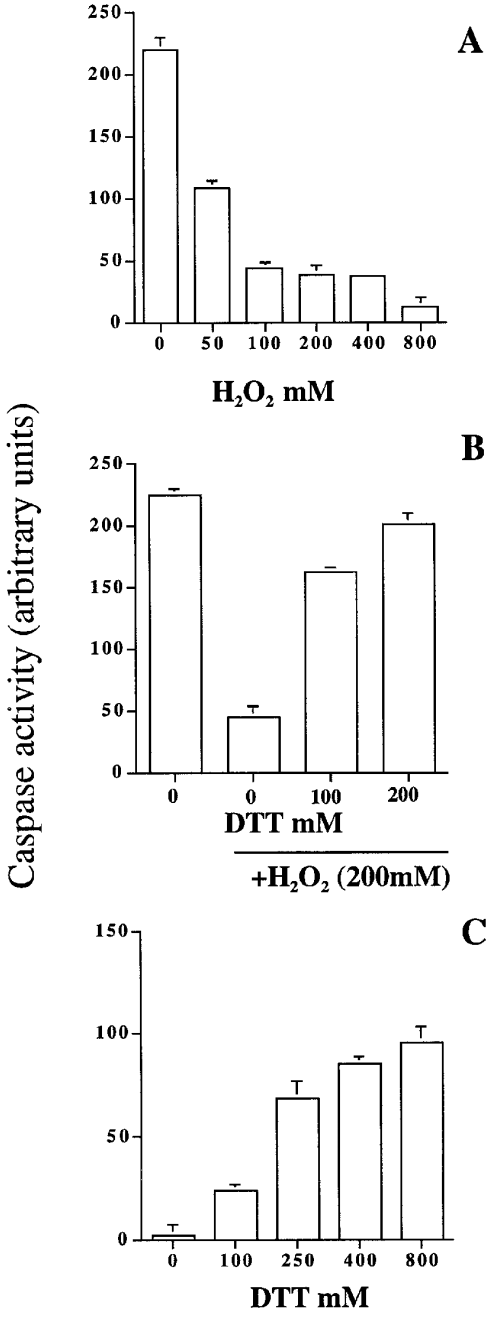

Figure 4 Effect of redox-active compounds on the caspase-3 (-like) activities of Fas treated Jurkats and retinal cell cultures. (**A**) Jurkat cells were treated with 300 ng/ml anti-human Fas IgM for 4 h and then lysed. Lysates were incubated with the indicated concentration of hydrogen peroxide (50 to 800 mM) for 30 min at 4°C followed by an assay for caspase-3(-like) activity involving the measurement of *p*NA release from the substrate DEVD-*p*NA. (**B**) Peroxide treated (200 mM for 30 min at 4°C) apoptotic Jurkat cell lysates were then incubated with the indicated concentration of the thiol reducing agent DTT (100 and 200 mM for 30 min at 4°C) and caspase-3 (-like) activity measured again. DTT reverses peroxide inactivation of caspase-3 (-like) caspases. (**C**) Retinal cells, cultured for 12 h, were lysed in an identical manner and incubated with the indicated concentration of DTT (100 to 800 mM for 30 min at 4°C) before caspase-3 (-like) activity was measured. Addition of DTT significantly restored caspase-3 (-like) activity in lysates taken from retinal cells undergoing cell death. These results are representative of three separate experiments ± S.E.

Table 1 Effects of antioxidants on retinal cell death *in vitro*

Treatment	% Cell death at 12 h
None	55±6
PDTC (40 μM)	6±5
$ZnCl_2$ (500 μM)	4±5

Percentage cell death was determined by measuring phosphatidylserine exposure using FITC conjugated annexin-V as described in Materials and Methods

been proposed to be an early event in apoptosis, however it appears that mitochondrial depolarisation is not necessary for apoptosis in all cell types. In this study, loss of mitochondrial membrane potential, as monitored by the mitochondrial specific probe JC-1 (Figure 5A), occurs after a significant ROS generation during retinal cell apoptosis. The kinetics of mitochondrial depolarisation appear to follow more closely other apoptotic events such as PS exposure (Figure 1A) and

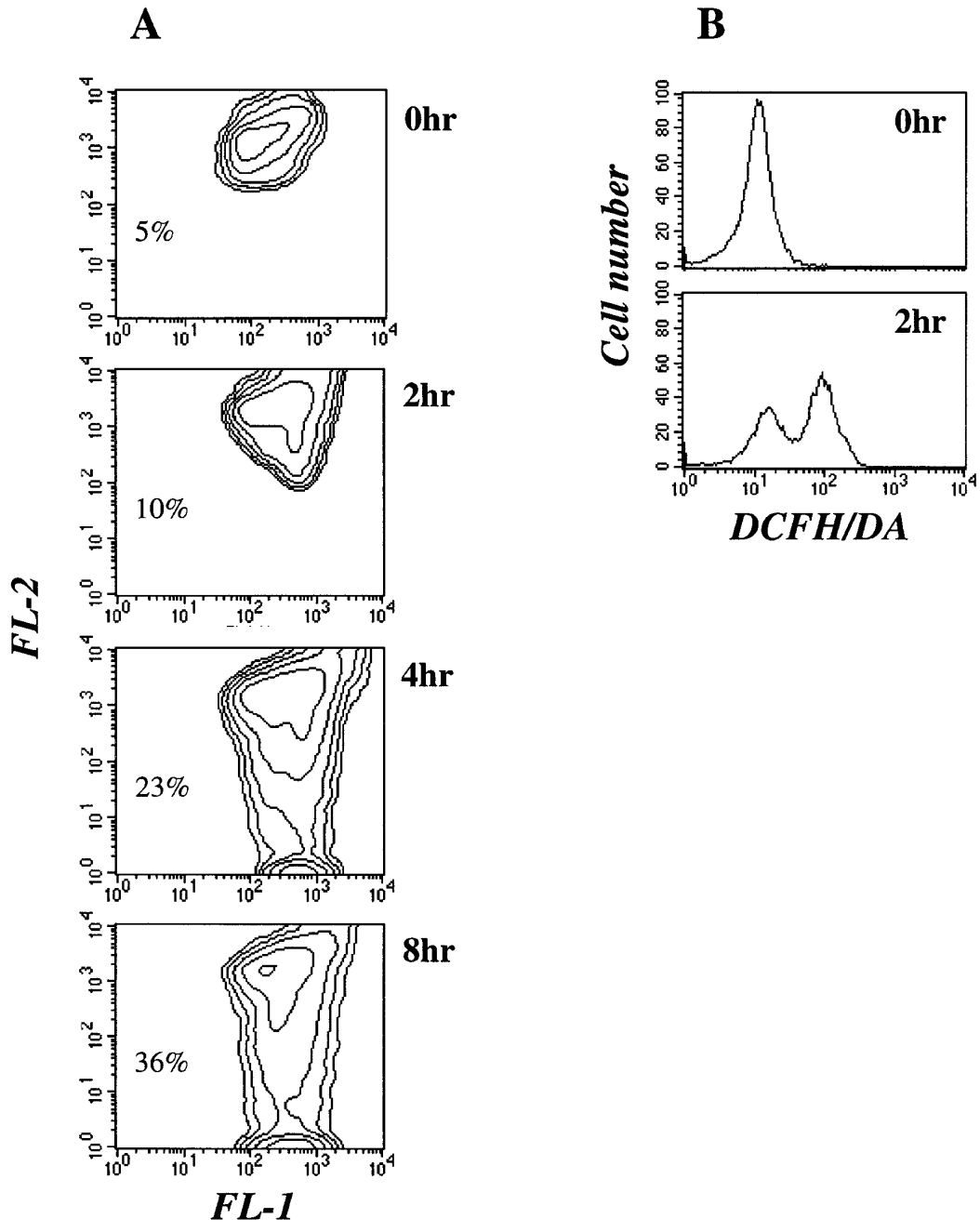

Figure 5 ROS generation precedes loss of mitochondrial membrane potential during retinal cell death *in vitro*. (**A**) Flow cytometric analysis of mitochondrial membrane potential in retinal cells as assessed by JC-1 fluorescence. JC-1 fluorescence in the FL-1 channel increases as mitochondrial membrane potential drops while its fluorescence in the FL-2 channel decreases. Percentage numbers indicate proportion of retinal cells with depolarised mitochondria at 2, 4, and 8 h, and immediately after isolation (0 h). (**B**) Intracellular ROS levels of retinal cells immediately after isolation (0 h) and following 2 h of culture were measured using the fluorescent probe DCFH/DA. Increases in fluorescence due to DCFH/DA indicates increased intracellular ROS. These results are representative of three individual experiments

cell shrinkage (Figure 1B). These data suggest that mitochondrial membrane depolarisation may result from direct ROS/oxidative damage to the mitochondria rather than the action of caspases.

Discussion

Despite the central role attributed to the caspases in apoptosis, emerging evidence now suggests that this family of proteases may not control the cellular commitment to programmed cell death in all cells. Caspase-independent cell death has now been described in several models of apoptosis including glucocorticoid induced thymocyte cell death,[30] growth factor withdrawal in haematopoietic cell death,[32] etoptoside,[31] ceramide[42] and nitric oxide (NO) induced death,[36] as well as cell death due to the enforced expression of Bax,[43] Bak,[34] c-Myc,[34] the adenovirus death factor E4orf4[44] and CD45[45] crosslinking. In many of these cases the use of caspase inhibitors prevented most features of apoptotic cell death, but several morphological and biochemical markers of apoptosis, including PS exposure,[44,46] loss of mitochondrial transmembrane potential,[31,42–45] chromatin condensation,[43,44,47,48] membrane blebbing[34,44] and cytoplasmic shrinkage[44,48] were retained. These studies have demonstrated that, at least in these models, several apoptotic events are not regulated by the caspases, and that while caspase activity may be sufficient for apoptotic cell death, it is not always necessary. This study describes a retinal cell apoptosis pathway *in vitro* which does not involve caspase activity. The lack of caspase activity as these cells die is evidenced by the lack of caspase-3 activation (Figure 2), the absence of PARP cleavage (data not shown) – a substitute for several members of the caspase family[49] – as well as the failure of several, broad spectrum caspase inhibitors to prevent or alter the characteristics and kinetics of the cell death observed (Figure 2 and data not shown).

The retinal cell death described in this study retains key characteristics of apoptotic cell death in the absence of caspase activity. These include the externalisation of PS to the outer leaflet of the plasma membrane, mitochondrial depolarisation, DNA strand nicking and cell shrinkage. The early detection of increased ROS generation here, and the previous demonstration that ROS act as mediators of photoreceptor apoptosis *in vitro*,[38] suggest that the caspase-independent apoptosis observed in retinal cells *in vitro* is driven by oxidative stress. This is further supported by the ability of antioxidants to prevent retinal and photoreceptor apoptosis *in vitro*[38] (and Table 1). Although PS exposure on the outer plasma membrane has been reported to be dependent on caspase activity,[50] alternative regulatory mechanisms for this process have been proposed. Indeed, studies using erythrocytes have shown the specific externalisation of PS induced by oxidative stress,[51] while in haematopoietic and lymphocytic cells direct PS oxidation has been observed prior to externalisation during apoptosis.[52,53] These reports provide evidence for the regulation of PS exposure and subsequent cell corpse engulfment by ROS, and suggest a mechanism for the PS exposure observed in the absence of caspase activity during retinal cell death *in vitro*.

The absence of low molecular weight, internucleosomal DNA fragmentation in retinal cell death *in vitro* may be associated with the apparent role of caspases in this apoptotic event. The only apoptosis specific pathway for the internucleosomal fragmentation of nuclear DNA described to date involves a caspase activated DNase (CAD) whose endogenous inhibitor (ICAD) must be cleaved in a caspase dependent manner.[28] The lack of low molecular DNA fragmentation observed in this study may be due to the absence of caspase activity during cell death. The labelling of retinal nuclei using the terminal dUTP nick end labelling assay, however, indicates the presence of DNA nicks or possibly high molecular weight DNA fragmentation during cell death.[54] It is possible that other endonucleases, not under the direct regulation of the caspases, are also involved in the initial stages of DNA degradation during programmed cell death.[55]

The loss of mitochondrial membrane potential in retinal cell death *in vitro* follows other events such as ROS generation and PS exposure. Loss of mitochondrial membrane potential has been ascribed a central role in the apoptotic process and has been proposed to act as a forward feeding loop for the activation of the downstream caspases (i.e. those containing short prodomains such as caspase-3, -6, and -7) through release of cytochrome c from the mitochondrial intermembrane space.[56] Active caspases may induce further mitochondrial alterations, including membrane rupture and the release of additional caspases and caspase activating factors culminating in a caspase activity amplification step.[57] The lack of caspase activity in the retinal cell death discussed here appears to relegate mitochondrial depolarisation to a later stage in the death pathway, possibly as a result of direct oxidative damage. Thus, the role of mitochondria as primary mediators of programmed cell death would appear to be dependent upon the ability of a given cell or stimulus to initiate caspase activation.

In this present study we have demonstrated that the failure of caspase inhibitors to prevent or alter retinal cell apoptosis *in vitro* is due to the lack of caspase activation during the death process in the first instance. The previously established role for ROS and loss of intracellular antioxidant defence such as glutathione in retinal cell apoptosis *in vitro*[38] may provide an explanation for the lack of caspase activation during programmed cell death in this model. The presence of an essential thiol group in the active site of the caspases would be predicted to render these enzymes redox sensitive.[58] The ability of DTT, a thiol reducing agent, to restore caspase activity in retinal cell lysates, as shown here, strongly suggests that caspase inactivation in this model is due to oxidation of the cysteine residue in the active site. This indicates that increased ROS generation during retinal cell death *in vitro* prevents activation of the caspases but allows the activation of a caspase-independent apoptotic pathway.

This study and other studies have shown the redox modification of caspase activity through the use of oxidants such as peroxide[40] (and Figure 4), diamide[59] and the dithiocarbamate disulfides.[60] It is becoming evident that the

cellular reducing environment maintained by endogenous antioxidants such as glutathione and thioredoxin[59] plays an important role in the ability of the caspases to execute the effector stages of the apoptotic program.

In conclusion, this study provides evidence for ROS driven, caspase-independent apoptosis, retaining key features such as externalisation of PS, cell shrinkage and DNA nicking. The failure of caspase inhibitors to prevent apoptosis suggests that therapeutic intervention at the level of the caspases may not be sufficient to prevent apoptosis in retinal degenerations such as RP. It has previously been reported that the baculoviral caspase inhibitor, p35, was not only capable of preventing apoptosis in retinal degenerative *Drosophila* mutants, but also of maintaining visual functionality.[61] Interestingly, a recent report has attributed an anti-oxidant property to p35[62] suggesting that cell survival in these retinal degenerative mutants may not only require inhibition of the effectors of the cell death machinery, but also of the initiating signals. In light of this view, and as our findings demonstrate, the use of anti-oxidants may be of more therapeutic benefit in the prevention of retinal degeneration in RP than inhibitors of the caspases.

Materials and Methods

Tissue culture

Adult C57/BL mice were used in all experiments. Retinal dissection and cell culture preparation were carried out according to the method previously described.[37] Briefly, enucleated eyes were placed in Hanks' basal salt solution (HBSS) and any mesenchyme removed. Using a watchmakers forceps, the choroid, sclera and pigmented epithelium were removed. The retina was then separated from the vitreous and lens and placed in cold medium. Tissue dissociation was achieved in a 0.25% trypsin solution (Gibco-BRL, Paisley, UK). Cells were seeded in multi-well plates and cultured in a previously described chemically defined Dulbecco's modified eagles (DME) medium[37] (high pyruvate, low glucose) (Gibco-BRL, UK).

Cell lines/reagents

Jurkat T-cells were maintained in RPMI containing 10% FCS. 32D cells were cultured in RPMI containing 10% FCS and 10% WEHI conditioned media. Agents used to induce apoptosis were anti-human Fas (300 ng/ml) (Upstate Biotech New York, USA) and exposure to ultraviolet (UV) irradiation (10 min). Caspase inhibitors employed were zVAD-fmk (Enzyme System Products, CA, USA), DEVD-CHO (Bachem, Essex, UK) and BD-fmk (Bachem, Essex, UK).

Measurement of PS exposure

The exposure of PS on the extracellular surface of the plasma membrane was monitored by the binding of Annexin-V-FITC following the manufacturer's instructions (BenderMed Systems, Vienna, Austria). Cells were simultaneously stained with 50 μg/ml propidium iodide (PI) prior to analysis on a FACScan flow cytometer (Becton Dickinson, USA) using an excitation of 488 nm. Fluorescence due to FITC and PI was measured at 530 and 590 nm respectively. Low fluorescence debris was gated out prior to analysis.

Assessment of DNA integrity

Double stranded DNA fragmentation was examined as previously described.[63] Briefly, cells (1×10^6/ml) were pelleted by centrifugation at $200 \times g$ for 5 min at room temperature. The pellets were resuspended in lysis buffer (20 mM EDTA, 100 mM Tris (pH 8.0), 0.8% (w/v) sodium lauryl sarcosinate) and 10 μl RNase A (1 mg/ml in 0.1 M sodium acetate, 0.3 mM EDTA pH 4.8) and incubated at 37°C for 1.5–2 h. Ten μl of proteinase K (20 mg/ml in distilled water) was then added and extracts incubated overnight at 50°C. Electrophoresis was carried out using 1.5% agarose gels and DNA was visualised under UV light following staining with ethidium bromide.

Terminal deoxy-uridine (dUTP) nick-end labelling (TUNEL)

In situ terminal dUTP nick end labelling was performed as previously described.[37] Briefly, cells were fixed in 1% para-formaldehyde and stored overnight at −20°C in 70% ethanol. After washing in phosphate buffer saline (PBS), cells were re-suspended in 50 μl of reaction mixture containing 0.1 mM dithiothreitol (DTT), 0.05 mg/ml BSA, 2.5 mM $CoCl_2$, 0.4 mM bio-16-dUTP and 0.1 U/ml terminal deoxynucleotidyl transferase (TdT) in 0.1 M Na cacodylate (pH 7.0) buffer. This mixture was incubated at 37°C for 30 min. Cells were then washed in PBS and re-suspended in 100 μl of staining buffer containing 2.5 mg/ml fluoresceinated avidin, 4× concentrated saline-sodium citrate buffer (0.3 M), 0.1% Triton X-100 and 5% (w/v) low-fat dried milk. Cells were then incubated for 30 min at room temperature in the dark before washing in PBS. Bio-16-dUTP and TdT enzyme from Boehringer Mannheim (Mannheim, Germany). Samples were read on a Becton Dickinson FACScan using excitation and emission wavelengths of 488 and 530 nm respectively.

Nuclear morphology analysis

Cells were stained in a 1 μg/ml DAPI/methanol (Sigma, Dublin, Ireland) solution for 15 min at 37°C. Following washing in PBS, cells were mounted in mowiol (Calbiochem, Nottingham, UK) and viewed under a fluorescence microscope (Nikon Eclipse E600) using a DAPI filter (excitation 340–380 nm, emission 435–485 nm).

Western blot analysis

Anti-caspase-3 rabbit polyclonal antibody obtained from Upstate Biotech (New York, USA) was used for immunoblot detection of caspase-3. Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride) containing antipain (1 μg/ml), aprotinin (1 μg/ml), chymostatin (1 μg/ml), leupeptin (0.1 μg/ml), pepstatin (1 μg/ml) and PMSF (0.1 mM). Equivalent amounts of protein, as determined by the Bio-Rad Protein Assay (Bio-Rad, Hemel Hempstead, UK) using bovine serum albumin as a standard, were resolved using SDS–PAGE followed by transfer to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membrane was blocked using phosphate buffered saline (PBS) containing 5% fat free dried milk before incubation with anti-caspase-3 antibody (1:1000) overnight. Blots were washed with PBS containing 0.01% Tween 20 (PBS-T), incubated with peroxidase conjugated secondary antibody, and washed once more with PBS-T. Membrane development was achieved using Enhanced Chemiluminescence (ECL) (Amersham, Buckinghamshire, UK).

Determination of DEVD-*p*NA cleavage

The measurement of DEVD-*p*NA (Calbiochem, Nottingham, UK) cleavage was performed in a spectrophotometric assay by monitoring the liberation of *p*NA due to caspase activity. Cells were lysed using a non-denaturing buffer (10 mM HEPES pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cell lysates were then incubated in an equal volume of 2$\times$ reaction buffer (50 mM HEPES pH 7.4, 0.5% CHAPS, 25 mM DTT) and 50 μM DEVD-*p*NA substrate at 37°C in a microtiter plate. Cleavage of the peptide substrate DEVD-*p*NA was monitored by *p*NA liberation in a SpectraMax-340 plate reader (Molecular Devices, CA, USA) by measuring absorption at 405 nm.

Analysis of intracellular ROS generation

Peroxide levels were determined using the previously described method of Hockenbery and co-workers.[64] Briefly, cells (5×10^5) were loaded with 5 μM DCFH/DA (Molecular Probes, Netherlands) for 1 h at 37°C prior to measurement on a Becton Dickinson FACScan flow cytometer with excitation and emission settings of 488 and 530 nm (FL-1) respectively.

Analysis of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane disruption was analyzed using JC-1 (Molecular Probes, Netherlands). JC-1 is a dual emission probe with increased fluorescence at 530 nm (FL-1) and reduced fluorescence at 590 nm (FL-2) associated with a decrease in mitochondrial transmembrane potential. JC-1 was used at 5 μg/ml from a 5 mg/ml stock prepared in DMSO. Cells were incubated with JC-1 for 15 min at 37°C and fluorescence measured in both FL-1 and FL-2 channels on a Becton Dickinson FACScan with an excitation of 488 nm.

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