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Apoptosis and its suppression in hepatocytes culture

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Abstract

In order to achieve the goal of developing extracorporeal liver support devices, it is necessary to optimise bioprocess environment such that viability and function are maximised. Optimising culture medium composition and controlling the constitution of the cellular microenvironment within the bioreactor have for many years been considered vital to achieving these aims. Coupled to this is the need to understand apoptosis, the prime suspect in the demise of animal cultures, including those of hepatocytes. Results presented here show that absent nutrients including glucose and amino acids play a substantial part in the induction of apoptosis. The use of chemical apoptosis inhibitors was utilised to investigate key components of hepatic apoptosis where caspases, predominantly caspase 8, were implicated in staurosporine (STS)-induced HepZ apoptosis. Caspase 9 and 3 activation although recorded was of less significance. Interestingly, these results were not consistent with those of mitochondrial membrane depolarisation where inhibition of caspase activation appeared to drive depolarisation. Inhibition of mitochondrial permeability transition and use of anti-oxidants was unsuccessful in reducing apoptosis, caspase activation and mitochondrial membrane depolarisation. In further studies, the anti-apoptotic gene *bcl-2* was over-expressed in HepZ, resulting in a cell line that was more robust and resistant to death induced by glucose and cystine deprivation and treatment with STS. Bcl-2 did not however show significant cytoprotectivity where apoptosis was stimulated by deprivation of glutamine and serum. Overall, results indicated that although apoptosis can be curbed by use of chemical inhibitors and genetic manipulation, their success is dependent on apoptotic stimuli.

Introduction

Liver transplantation is currently the only treatment for end-stage liver disease and congenital hepatic disorders. However, the shortage of organ donors results in high mortality rates. Thus, much research attention has focussed on development of extracorporeal liver support devices, in which cultured hepatocytes carry out the multitude of functions of the liver. A major bottleneck in achieving this goal is the sheer number of viable hepatocytes that would be required (Bratch and Al-Rubeai 2001). As with other cultured cells, a stumbling block in reaching high-density cultures whilst retaining viability is the occurrence of apoptosis or programmed cell death (PCD) within these intensive culture systems.

Apoptosis is characterised by a number of morphological and biochemical changes including cell shrinkage, DNA fragmentation, membrane

blebbing, proteolytic degradation of cytoskeletal proteins, decrease in mitochondrial membrane potential ($\Delta\psi_m$), alteration in the ratio of pro- and anti-apoptotic proteins and activation of caspases. Although early interest in apoptosis stemmed from cancer research, the discovery of apoptosis in hybridoma cultures highlighted the significance of apoptosis to the biotechnology industry (Al-Rubeai et al. 1990). Within the bioreactor environment apoptosis can be triggered by a number of stimuli, including exhaustion of essential nutrients (amino acids, serum components, and glucose), accumulation of metabolic by-products, and alterations in pH, dissolved oxygen content and medium osmolarity (Singh et al. 1994; Simpson et al. 1998; Ishaque and Al-Rubeai 1999, 2002). Additional environmental factors that lead to apoptotic cell death in large-scale mammalian cell cultures include those caused by mechanical stresses (Al-Rubeai et al. 1995).

Caspases are a family of 14 cysteine proteases that are expressed as zymogens (procasapes), requiring proteolytic cleavage at specific aspartic acid residue for activation. These are categorised into initiator (upstream) and effector or downstream caspase. Caspase 8 initiates the extrinsic death-receptor pathway, which is stimulated by binding of tumour necrosis factor alpha (TNF-α) and Fas to their cognate ligands tumour necrosis factor 1 (TNFR1) and FasL, respectively. The resulting conformational change in TNF-α and Fas facilitates binding of procaspase 8 and its activation to caspase 8. The intrinsic mitochondrial-mediated pathway is initiated by caspase 9, which is activated upon release of cytochrome *c* from the mitochondria. In the presence of deoxyadenosine triphosphate (dATP), cytochrome *c* binds with apoptosis protease-activating factor-1 (Apaf-1) to form the apoptosome. The resulting conformational change in Apaf-1 and subsequent localisation of procaspase 9 leads to formation of active caspase 9. Caspase 8 and caspase 9 proceed to activate downstream effector caspases (caspases $-3, -6$ and -7), culminating in the degradation of a number of substrates, including poly (ADP-ribose) polymerase (PARP) and DNA fragmentation factor (DFF) and physical damage of the cell.

The function of mitochondria is not limited to production adenosine triphosphate (ATP) *via* glycolysis and oxidative phosphorylation in the inner mitochondrial membrane. The outer mitochondrial membrane is implicated in cell signalling and modulation of cell death (Skulachev 1996; Susin et al. 1998; Griffiths 2000; Parone 2002). Stimuli such as UV radiation, chemotherapeutic agents and increase in reactive oxygen species (ROS) have been found to induce apoptosis *via* the intrinsic pathway (Gupta 2001). Research suggests alterations in mitochondrial membrane potential ($\Delta\psi_m$) occurs as an early event of apoptosis (Zamzami et al. 1995; Kroemer et al. 1998). The location of members of the Bcl-2 family within the intermembrane space of mitochondria further supports the involvement of mitochondria in the apoptotic process. Recent studies have however cast doubt on the involvement of mitochondria on regulating apoptosis reporting that cytochrome *c* release is not necessary for cell death and that its release is not always facilitated by mitochondrial membrane depolarisation (Hatano et al. 2000). Further still, there are suggestion that mitochondrial membrane depolarisation is in itself capable of inducing caspase activation and thus apoptosis (Salvioli et al. 2000).

Strategies to control cell death have included medium (nutritional) optimisation and chemical and genetic methods. Chemicals such as anti-oxidants, mitochondrial permeability transition (MPT) inhibitors and caspase inhibitors have been used to suppress apoptosis in mammalian cell cultures. Anti-oxidants work primarily by neutralising ROS and effectively suppress apoptosis in mammalian cell culture (Mastrangelo 1999). *N*-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) have been shown to extend the survival of human neurons, HL-60 cells and murine L929 cells under adverse culture conditions (Mayer and Noble 1994; Talley et al. 1995; McGowan et al. 1996). However, the anti-oxidant cyclosporin A (CsA) has been shown to protect against nitric oxide-induced apoptosis is short-lived, with demise in permeability transition (PT) within an hour, perhaps suggesting inhibition of MPT (e.g. with bongkrekic acid; BA) would more effective protection from apoptosis is transitory (Zamzami et al. 1996).

Due to their central role in apoptosis caspases are an obvious target for studies in apoptosis suppression, indeed a number of synthetic peptide inhibitors such as *N*-benzyloxycarbonyl-Val-Ala-Asp-flouromethylketone (z-VAD.fmk), Z-Tyr-Val-

Ala-Asp(OMe)-fluoromethyl ketone (Y-VAD-FMK) and acetyl-Asp-Glu-ValAsp-aldehyde (Ac-DEVD-CHO) are the most widely used, whilst *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-flouromethylketone (z-IETD-FMK) and *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-flouromethylketone (z-LEHD-FMK) have been shown to inhibit the activity of caspase 8 and caspase 9, respectively. Y-VAD-FMK and Ac-DEVD-CHO have been used to block apoptosis in response to a variety of stimuli including serum and growth factor withdrawal, irradiation and presence of anti-Fas antibody. z-VAD-FMK is a broad-spectrum caspase inhibitor is used most extensively in research and is shown to have wide-ranging effects (reviewed by Mastrangelo 1999). Of considerable interest is the finding that z-VAD-FMK not only inhibits cell death in adenovirus-infected HeLa cells but resulted in augmented virus production (Chiou and White 1998).

The majority of genetic strategies have concentrated on the Bcl-2 family of proteins, with the *bcl-2* proto-oncogene considered the prototype apoptosis suppressor. First described in non-Hodgkin's B-cell lymphoma, *bcl-2* was the first proto-oncogene shown to exert its effects on cell death rather then cell proliferation (Tsujimoto et al. 1984; Hockenberry et al. 1990). Members of the Bcl-2 family have been shown to comprise of pro-apoptotic (e.g. Bax, Bid, Bad) and anti-apoptotic (e.g. Bcl-2, Bcl-x_L, Bcl-w) members (Kroemer 1997; Blatt and Glick 2001; Budd 2001). Many theories exist on how Bcl-2 prevents caspase activation including dimerisation with pro-apoptotic members rendering them ineffective, preventing permeability transition (PT) pore formation on the outer mitochondrial membrane, thereby preventing cytochrome *c* release and unregulated influx and efflux of ions across these membranes (Antonsson et al. 1997; Rao and White 1997; Robb-Gasper et al. 1998). The Bcl-2 family has been implicated in other non-apoptotic roles including modifying cell cycle and cell growth profiles and increasing cell densities and specific productivity (O'Reilly et al. 1996; Huang et al. 1997; Vaux et al. 1998; Simpson et al. 1999). Bcl-2 has been extensively over-expressed in a number of cell lines including CHO, hybridoma, and myeloma cells and has been shown to suppress apoptosis, in response to many inducing agents (Singh et al. 1996, Simpson et al. 1997, 1998, 1999; Suzuki et al. 1997; Fussenegger et al. 2000; Tey et al. 2000a, b; Ishaque and Al-Rubeai 2002).

In this study apoptosis in a hepatocyte cell line derived from primary human hepatocytes, HepZ is investigated. We demonstrate that nutrient deprivation induces apoptosis and that this mode of cell death can indeed be suppressed by administration of chemical inhibitors including anti-oxidants, caspase inhibitors and inhibitors of MPT. The influence of Bcl-2 on growth and survival of HepZ in batch culture is also considered, with results confirming that Bcl-2 over-expression yields more robust cells whilst increasing culture longevity. Upon deprivation of key nutrients Bcl-2 over-expression is seen to provide cytoprotectivity, although not under all deprived conditions.

Materials and methods

Cell line and cell culture

The HepZ cell line was obtained from G. Jennings, HepaVec AG, Germany. HepZ were transfected with pEFpGKpuropA and pEFbcl-2Y28ApGKpuropA and are referred to as HepZ.pEF and HepZ.bcl2, respectively. The pEF and bcl-2 constructs were kindly provided by D. Huang, Royal Melbourne Hospital, Australia. All cells were cultured at 37 °C in Dulbecco's Modified Eagles Medium (DMEM; Gibco, UK) supplemented with 10% (v/v) foetal calf serum (FCS; PAA, UK). Cultures were maintained in vented T-25 or T-75 flasks in a humidified 95% air/5% CO_2 incubator. Inocula for all experiments were taken from the mid-exponential growth phase of these cultures.

Assessment of growth and viability

Cell number and viability were determined by standard trypan blue exclusion method. Early apoptotic (EA), late apoptotic (LA), viable (V) and necrotic (N) cell fractions were quantified by fluorescence microscopy. Cell suspensions were stained with equal volumes of dual staining solution containing acridine orange (AO, 10 μg ml^{-1}; Sigma) and propidium iodide (PI, 10 μg ml^{-1}; Sigma). Cells were classified by virtue of colour

and chromatin morphology as previously described (Simpson et al. 1999).

Determination of caspase activity

Activation of caspases was determined using CaspaTag^{TM} Fluorescein Caspase (VAD) Activity Kit (Intergen, UK). Manufacturer instructions were followed. Briefly, 10 μl FAM-VAD-FMK (30× solution) was added to 300 μl of cell suspension (10^6cells ml^{-1}), and incubated at 37 °C under 5% CO_2 for 60 min, in the dark. Pellets were washed twice before resuspension in wash buffer. Samples were placed on ice before FACS analysis (emission at 532 nm after excitation at 488 nm).

Assessment of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was analysed using 5, 5′-6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolecarbocyanine iodide (JC-1; Molecular Probes). JC-1 is a dichromatic dye that exists in monomeric form at low $\Delta\psi_m$ and emitting green fluorescence after excitation at 488 nm. At high $\Delta\psi_m$, J-aggregates are formed, emitting red fluorescence. Ratiometric analysis of green/red fluorescence provides information on mitochondrial activity.

JC-1 (5 μg ml^{-1}) was added to 5×10^5 cells in a 1 ml volume of medium. Cells were incubated at 37 °C for 15 min and washed in PBS before FACS J-monomers and J-aggregates were detected at 527 nm and 590 nm, respectively after excitation at 488 nm.

Annexin V-FITC/PI dual staining

Translocation of phosphatidylserine was measured by using the high affinity Ca^{2+} dependent phospholipid binding protein, Annexin-V conjugated to fluorescein isothiocyanate (FITC; Catlag MedSystems, UK).

10^6 cells were harvested and washed once in PBS before being resuspended in 190 μl binding buffer (10 mM Hepes/NaOH, pH7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$). Annexin V-FITC was added to the cell suspension (to a final concentration of 2.5 μg ml^{-1}) after which cells were incubated in the dark at room temperature for 10 min. PI (50 μg ml^{-1}) was added 5 min before analysis. Fluorescence emission was measured at 525 nm and 610 nm after excitation at 488 nm.

Preparation of amino acid deprived medium

Amino acid deprived medium was prepared closely following manufacturer guidelines (Gibco Life Technology, UK). Briefly, reconstituted vitamin mixture was added to MEM Balanced Salt Solution containing $NaHCO_3$ and dialysed FCS (PAA). Fourteen aliquots of equal volume were made before adding individual reconstituted amino acids, ensuring a different one of 13 amino acids was omitted from each preparation. One preparation contained all thirteen amino acids and was used as a control. The pH of each of the preparations was adjusted to pH 7.2 with 1 M HCl and 1 M NaOH.

Staurosporine (STS) treatment

Where HepZ are treated with STS, 5×10^5 cells were seeded into 6-well plates and incubated at 37 °C overnight before addition of STS to a final concentration of 2 μM. In experiments where anti-apoptotic agents were used, they were added 1 h before addition of STS.

Preparation of apoptosis inhibitor solutions

Stock solutions (100×) of caspase inhibitors were prepared in dimethyl sulphoxide (DMSO; Sigma) and diluted to the relevant working concentration in cell culture medium. Caspase inhibitors were purchased from Calbiochem (MERCK Biosciences Ltd). Bongkrekic acid solution and $ZnSO_4 \cdot 7H_2O$ were purchased from Sigma Aldrich. $ZnSO_4 \cdot 7H_2O$ was dissolved in distilled water. Inhibitors were used at the following concentrations; z-IETD-FMK (IETD; 75 μM); z-LEHD-FMK (LEHD; 75 μM); Ac-DEVD-CHO (DEVD; 100 μM); z-VAD-FMK (zVAD; 100 μM);

bongkrekic acid (BA; 10 μM) and $ZnSO_4$ś$7H_2O$ (Zn^{2+}; 100 μM).

Results

Nutrient deprivation

Glucose is a vital component for the production of energy *via* the glycolytic pathway hence; it was of interest to investigate its role in the prevention of apoptosis. 2×10^5 cells were inoculated in glucose-free DMEM supplemented with dialysed FCS. Cell viability was determined by AO/PI dual staining. The absence of glucose was seen to inhibit cell proliferation, although cell viability was maintained without any observable drop for 96 h of the batch culture. Thereafter, a dramatic fall in viable cell number and was observed with apoptosis being the predominant cause of cell death. In the absence of glucose 90% apoptosis was recorded compared to 60% in control cultures (Figure 1).

Amino acids play a crucial role in biosynthesis and metabolic reactions as well as having an influence on some regulatory pathways; in concert with hormones, amino acids are involved in gene expression and signal transduction. Studies carried out in hybridoma culture were mimicked in HepZ culture where the effect of deprivation of a single amino acid on induction of HepZ apoptosis was investigated (Simpson et al. 1998). Stock cultures from mid-exponential pellets were resuspended in amino acid deprived medium and seeded onto

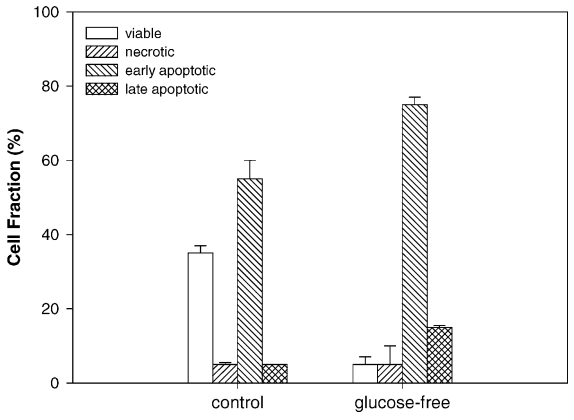

Figure 1. Cell death in glucose deprived medium after 120 h of batch culture.

6-well plates to a final density of 2×10^4 cells ml^{-1}. Deprivation of amino acids affected HepZ with varying degrees of severity, although the general finding was that deprivation of amino acids leads to apoptotic rather than necrotic cell death (Figure 2). Deprivation of the amino acids arginine, tyrosine and lysine had little effect on cell death with the fraction of viable cell population above 90% retained after a 48 h deprivation period (Figure 2a). Arginine and histidine continued to have little effect on apoptosis induction after a further 48 h of deprivation (Figure 2b). Surprisingly, glutamine, a vital nucleotide precursor was seen to have limited affect with 80% viability recorded after 96 h of deprivation (Figure 2b). On the other hand, other amino acids, in particular cystine, had considerable influence on induction of apoptosis. Within the first 48 h of cystine deprivation 57.3% of the population exhibited classic apoptotic morphology such as chomatin condensation, as observed by fluorescence microscopy. Within 96 h, no cellular structures could be visualised by fluorescence microscopy, suggesting complete cellular destruction. The study was repeated using freshly isolated (primary) rat hepatocyte. Unsurprisingly primary cells were more sensitive to deprivation, with cystine deprivation leading to complete destruction of the cell population within 48 h (data not shown). Again, the predominant trend was that amino acid deprivation lead to apoptosis.

Products of cellular metabolism within culture medium are equally destructive to cell cultures if allowed to accumulate within the growth medium. During HepZ batch culture, the highest concentration of accumulated ammonia recorded was 7.4 mM (data not shown). In order to determine whether the levels of ammonia accumulated during HepZ batch culture were sufficient to induce cell death, the effect of elevated ammonia levels were investigated. Figure 3 illustrates the findings after 3 days of batch cultures, where medium ammonia levels were raised to 7 and 14 mM by addition of ammonium sulphate, medium pH was corrected to pH 7.2 by addition of HCl (1 M). Results suggest that these levels were neither toxic nor sufficient to induce premature cell death in HepZ when compared to the untreated control cultures. In fact, little difference in culture progression was recorded. In all cultures (treated and untreated), viability was maintained above 90%.

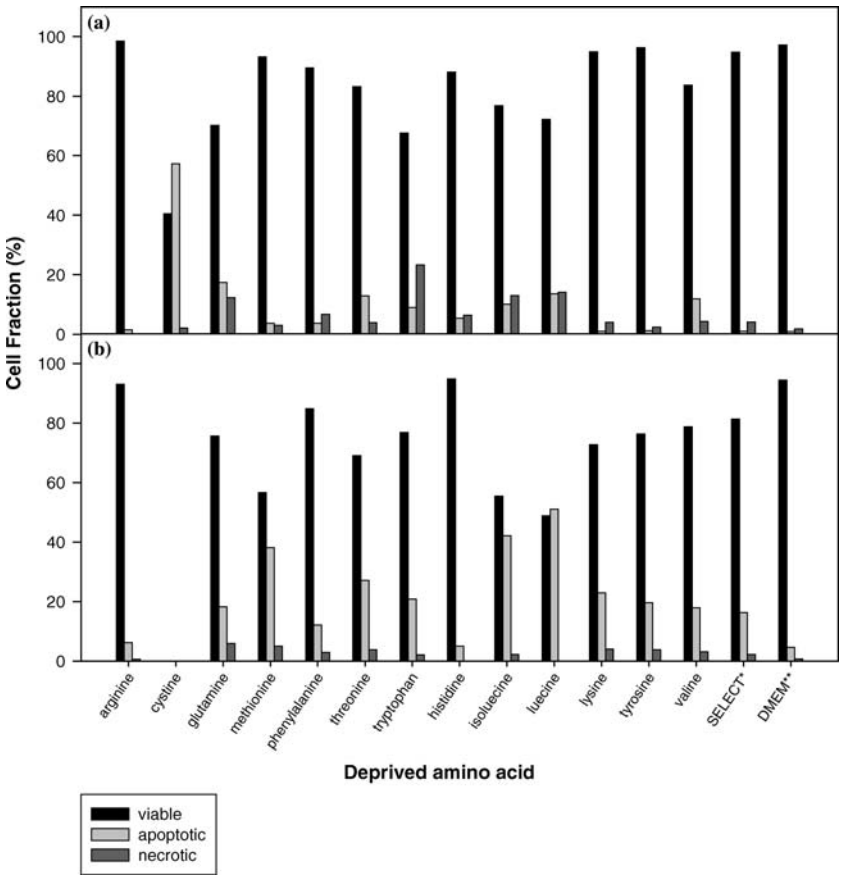

Figure 2. Induction of cell death in HepZ after 48 h (a) and 96 h (b) deprivation of single amino acid. DMEM** denotes DMEM + 10% *d* FCS and SELECT* denotes SELECTAMINE with all amino acid constituents + 10% *d*FCS. Data represents mean apoptosis levels in thee plates after a 96 h incubation period. Data represents the average of three independent experiments.

Chemical inhibition of HepZ apoptosis

It is not only important to identify the occurrence of apoptosis in cell culture systems; but knowledge of the mechanism(s) and machinery of apoptosis is a valuable tool in inhibiting the apoptotic process. In this study, an attempt was made to decipher the machinery and elucidate the pathways(s) of apoptosis in HepZ. In order to investigate the mechanism(s) of apoptosis in HepZ, the protein kinase inhibitor STS was used to induce this mode of cell death. This was achieved by utilising several apoptosis inhibitor that had previously been shown to prevent apoptosis in a variety of mammalian cell lines. It was hypothesised that by inhibiting a particular stage of the apoptotic process the involvement of the associated 'apoptosis factor' could be inferred. Studies were carried out to determine the effect of STS treatment on activation of caspases and on alterations in mitochondrial membrane potential ($\Delta\psi_m$) (Figure 4). 5×10^5 cells were seeded into 6-well plates and incubated at 37 °C overnight before addition of STS to a final concentration of 2 μM. Control (untreated) cultures exhibited 15.7% mitochondrial membrane depolarisation whilst addition of STS increased this value to 58.7% (Figure 4b). Figure 5 shows series photographs taken from a time-lapse microscopy of untreated and STS treated HepZ during batch culture. In the control culture there is evidence of cell–cell interaction as cells spread to form a monolayer. Cells undergoing mitosis are seen to detach briefly from the tissue culture plastic as daughter cells are formed, immediately reattaching and continuing to spread. In contrast, in the STS-treated culture disintegration of the

monolayer is observed and is characterised by loss of interacellular interaction, with cell seemingly retracting from the monolayer. Release of cytoplasmic material into the extracellular matrix (medium) is also observed.

Studies were carried out to determine the effect of STS treatment on activation of caspases and on alterations in $\Delta\psi_m$. In STS treated culture, there was evidence that mitochondria within a presumably 'homogeneous' cell population behave heterogeneously, where some retain $\Delta\psi_m$, whilst in others mitochondrial membrane depolarisation occurs (Figure 4c). Within untreated cultures 15.7% of mitochondria exhibited the depolarised characteristics associated with apoptosis. Clearly, addition of STS did affect the proportion of mitochondria with the fraction of depolarised membranes increasing to 58.7%. With respect to caspase activation, 6.36% of control cells displayed some caspase activity whilst similar data for STS treated HepZ showed that 74.35% of the cell population had some degree of caspase activity. Mean FITC fluorescence increases from 0.37 channels in untreated cultures to 2.04 channels in STS-treated culture (Figure 4b). Figure 5b illustrates morphological changes in HepZ as they undergo STS-induced apoptosis. After 4 h there is evidence of cells becoming detached and complete destruction of the monolayer and cellular structures was observed after 14 h. On the other hand,

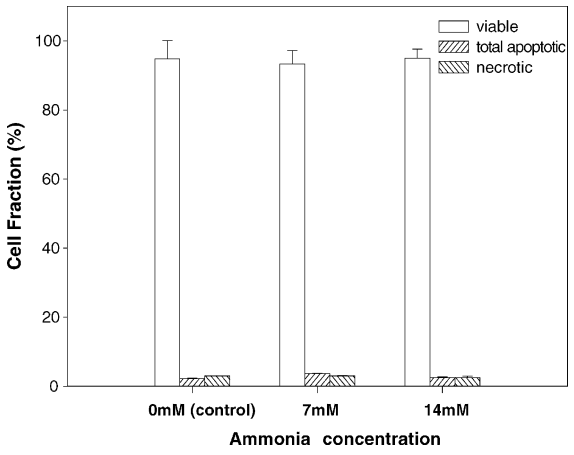

Figure 3. Survival of HepZ in the presece of elevated ammonia levels HepZ were cultured in DMEM supplemented with 10% FCS and 7 and 14 mM ammonium sulphate. Apoptosis levels were determined by AO/PI dual staining and fluorescence microscopy. Data shows mean ± S.D of thee replicates.

continued spreading of the monolayer is observed in the control culture (Figure 5a) with cellular detachment recorded prior to cell division.

Figure 6 shows the response of HepZ to STS treatment in the presence of four inhibitors of caspase activation: inhibitor of caspase 9 (using LEHD), inhibitor of caspase 8 (using IETD), inhibitor of caspase 3 (using DEVD) and broad spectrum caspase inhibitor (using zVAD). For each inhibitor, dosage studies were carried out to determine the optimum concentration of inhibitor to use (data not shown). Inhibition of caspase 9 activation (using LEHD; 75 μM) appeared not to prevent apoptosis, as determined by fluorescence microscopy more than 40% of the cell population exhibited classic apoptotic morphology, suggesting that activation of procaspase 9 is not directly triggered by STS. Similarly, inhibition of the downstream caspase, caspase 3, did not prevent apoptosis in HepZ as the viable cell fraction was reduced to below 60% in the presence of DEVD (100 μM). In stark contrast, inhibition of the activation of caspase 8 (by IETD; 75 μM) and use of the broad-spectrum caspase inhibitor, zVAD (100 μM) significantly reduced the occurrence of apoptosis. Where caspase 8 was inhibited, the apoptotic fraction was 5.3%. This finding supports the utilisation of STS to model death receptor-mediated apoptosis, the predominant pathway by which caspase 8 is activated. zVAD also showed cytoprotection; the level of apoptosis detected in HepZ was only 4.3%. However, the ability of bongkekric acid (BA) and Zn^{2+} (added as $ZnSO_4$) to inhibit STS-induced apoptosis in HepZ was not detected. Fluorescence microscopy revealed 45.8% apoptosis in the presence of BA and 47.2% for $ZnSO_4$, where STS cultures exhibited 44.8% of apoptotic cells.

Interestingly, Figures 7c–f show that regardless of how effective a particular caspase inhibitor is, with respect to preventing apoptosis, mitochondrial depolarisation still occurs. In the presence of all caspase inhibitors, more than 70% of the mitochondrial population exhibited increased mean fluorescence ratio. In the control cultures, 15.7% of mitochondria showed a similar increase whilst STS treated cultures showed 58.7% (Figure 7a and b). Coupled to mitochondrial membrane depolarisation, STS resulted in an increased mean FITC fluorescence (indicative of increased caspase activity) compared to control cultures

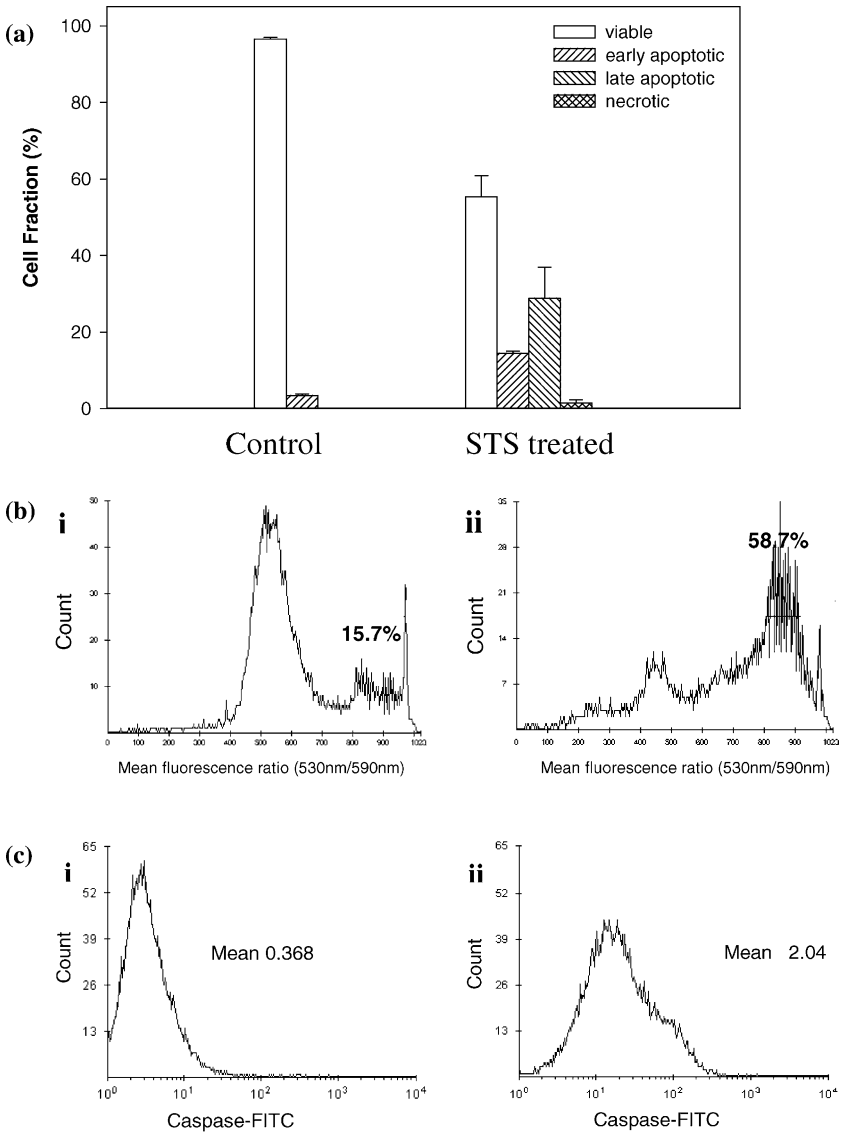

Figure 4. Affect of STS on apoptosis (a), mitochondrial membrane depolarisation (b) and caspase activation (c) in HepZ. (a) Apoptosis was measured by fluorescence microscopy. (b) Mitochondrial membrane depolarisation was measured by JC-1 and is depicted as cell count *vs*. J-ratio (530 nm/590 nm). Cell debris was removed from analysis on FS *vs*. SS plot and (c) caspase activity was determined using CaspaTagä Fluorescein Caspase (VAD) Activity Kit. Fluorescence was detected at 530 nm (Casp-FITC) after excitation at 488 nm. Data is presented as cell count *vs*. fluorescence at 530 nm (Caspase-FITC). (i) and (ii) represent control and STS treated cultures, respectively.

(Figure 8). There was however, correlation between prevention of apoptosis and reduced caspase activity. IETD and zVAD, the most protective agents showed lower mean fluorescence than other inhibitors, although still significantly higher than that in control cultures. The fluorescence of both DEVD and LEHD treated cultures was similar to that recorded in those treated with STS only. However, BA (an inhibitor of MPT formation) and the anti-oxidant Zn^{2+} (added as $ZnSO_4$) showed an excellent potency in blocking membrane depolarisation (Figure 7g and h). These findings are inconsistent with caspase activity data where it was observed that caspase activation in

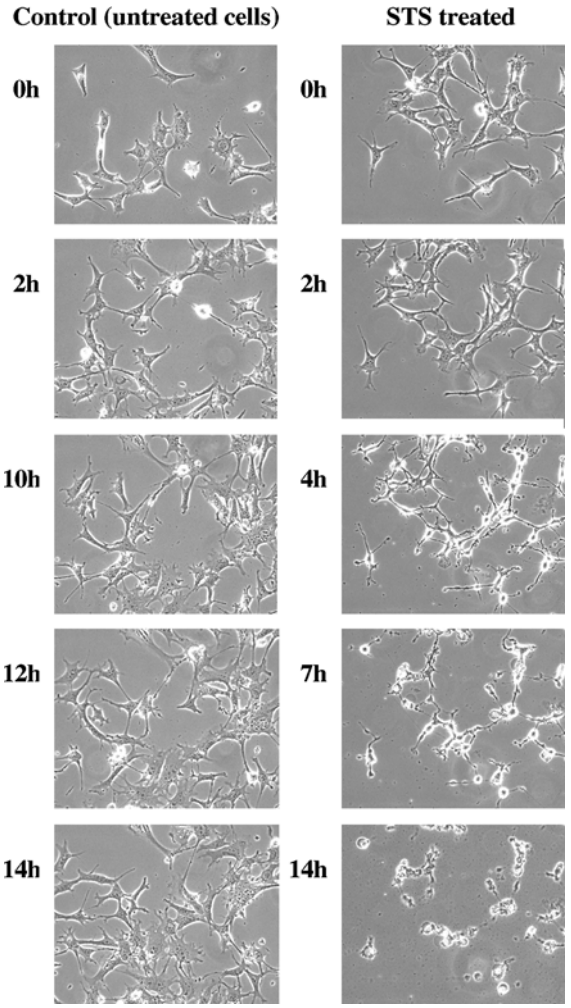

Figure 5. STS-induced apoptosis in HepZ. Images were generated from time-lapse microscopy (TLM). Untreated HepZ and STS treated HepZ were filmed every 2 min over an 18 h period using a Zeiss Axiovert 135TV microscope. A video clip is available upon request from the author.

the presence of BA and Zn^{2+} was comparable to that in their absence. BA and $ZnSO_4$ have affected mitochondrial membrane potential they have not reduced caspase activity and consequently have not protected the cells from the damaging effect of STS as shown in Figure 6.

Effect of genetic manipulation

The *bcl-2* plasmid in the transfected construct appeared to have little effect on maximum cell number achieved during batch culture -1.1×10^6 cells/ml for HepZ and 1.13×10^6 cells/ml for HepZ.bcl2 (Figure 9). Differences between cultures became apparent as cells entered the death phase (after 96 h) where the parental cells (HepZ) rapidly lose viability. In contrast, Bcl-2 over-expressing cells, HepZ.bcl2, retained viable cell numbers of 2.2×10^5cells/ml for a further 24 h (i.e. after 144 h of batch culture).

In experiments where cells were exposed to STS (2 μM) revealed that Bcl-2 did confer protection (Figure 10). Initial signs of apoptosis in the controls (parental cell line) were observed 4 h after exposure. After 8 h exposure apoptosis 50% (HepZ) showed hallmarks of apoptosis whilst HepZ.bcl2 retained viability at 89.77% compared to 98.3% in the untreated cultures. The ability of Bcl-2 to protect against nutrient deprivation was also investigated. After 72 h of culture, HepZ.bcl2 showed a lower level of apoptosis (28.2% and 22.5%), although the parental cell line had a lower proportion of mitochondria with depolarised membranes – 32.3% compared to 14.1% (Table 1 and Figure 11). However, under sub-optimal culture conditions (i.e. glucose, serum, glutamine and cystine deprivation) Bcl-2 was observed to prevent mitochondrial membrane depolarisation – 10% and 40% reduction in proportion of depolarised mitochondria upon glucose and serum deprivation, respectively. The most dramatic effect recorded in the absence of glutamine where the proportion of depolarise mitochondrial membrane fell from 76.3% in the parental line to 8.5% in Hep-Z (Figure 11). Whilst Bcl-2 successfully prevented mitochondrial membrane depolarisation, the results obtained were not coherent with those for apoptosis where cytoprotectivity was recorded only in the absence of glucose and cystine, with apoptosis levels falling from 60.5% to 21.2% and 48.5% to 27.5% (Table 1). In the absence serum and glutamine no significant effect was recorded. Notably, the incidence of necrosis was increased in all but serum-deprived cultures, with the most striking increment observed in glucose-deprived cultures where necrosis increased from 0.9% to 12.5% (Table 1).

Discussion

Culture medium is made of components that are deemed vital as precursors for biosynthetic

Table 1. Apoptotic and necrotic cell death in nutrient deprived batch culture of HepZ and HepZ.bcl2 measured at either 72 or 120 h as indicated.

	HepZ		HepZ.bcl2	
	Apoptotic (AV^+/PI^-)	Necrotic (AV^+/PI^+)	Apoptotic (AV^+/PI^-)	Necrotic (AV^+/PI^+)
Control (72 h)	28.2	1.1	22.5	4.2
Glutamine (72 h)	45.6	0.1	40.5	4.7
Glucose (72 h)	60.5	0.9	21.2	12.5
Serum (120 h)	51.7	1.8	57.5	0.4
Cystine (72 h)	48.5	0.1	27.5	3.3

Cells were stained with FITC-Annexin V and PI and fluorescence emissions at 530 m and 610 nm.

reactions or as effectors of cell proliferation and inhibitors of cell death. Thus, any limitation or deprivation of medium components will inevitably result in apoptotic or necrotic cell death.

Amino acids play a crucial role in biosynthetic and metabolic reactions as well as having an influence on some regulatory pathways. In concert with hormones, amino acids are involved in gene expression and signal transduction. In this study deprivation of amino acids affected HepZ with varying degrees of severity, although the general finding was that deprivation leads to apoptotic rather than necrotic cell death. Amino acids considered essential for cellular proliferation were not always revealed as essential for survival, in particular glutamine. This discovery was in stark contrast to reports describing the induction of apoptosis in the absence of glutamine from the culture of many cell lines (Mecille and Massie 1994; Singh et al. 1994; Simpson et al. 1998; Sanfelui and Stephanopoulos 1999). Only cells that endogenously express GS synthetase or those genetically engineered to do so would be expected to survive such conditions as they are able to convert glutamic acid and ammonia to glutamine. Although foetal hepatocytes contain GS mRNA, it is quickly lost after birth. The dramatic apoptotic effect of cystine was initially thought to be indicative of the crucial role of thiol compounds in regulating cell death. Sudden depletion of cystine (and glutamine) has been shown to lead to a decline in mitochondrial respiration, a phenomenon strongly associated with reduced Intracellular glutathione (GSH) levels, the most abundant thiol in hepatocytes. GSH protects cells from toxic insult and oxidative damage in a reaction catalysed

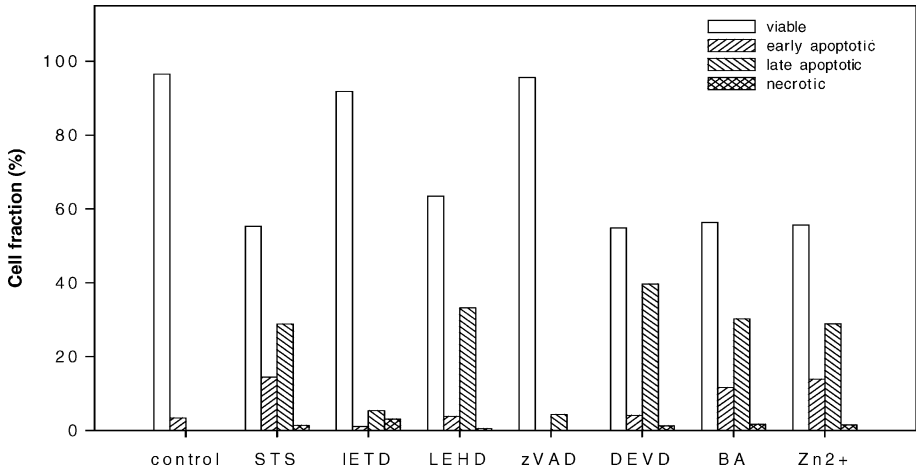

Figure 6. Chemical inhibition of STS-induced apoptosis. Mid-exponential phase were seeded into 6-well plates. After overnight incubation at 37 °C chemical inhibitors were added to medium to the following final concentrations – z-IETD-FMK(IETD; 75 μM); z-LEHD-FMK (LEHD; 75 μM); Ac-DEVD-CHO (DEVD; 100 μM); z-VAD-FMK (zVAD; 100 μM); bongkrekic acid (BA; 10 μM) and zinc sulphate (Zn^{2+}; 100 μM). Two hours after addition of inhibitors, STS (2 μM) was added. Apoptosis levels were determined by AO/PI staining 6 h after addition of STS.

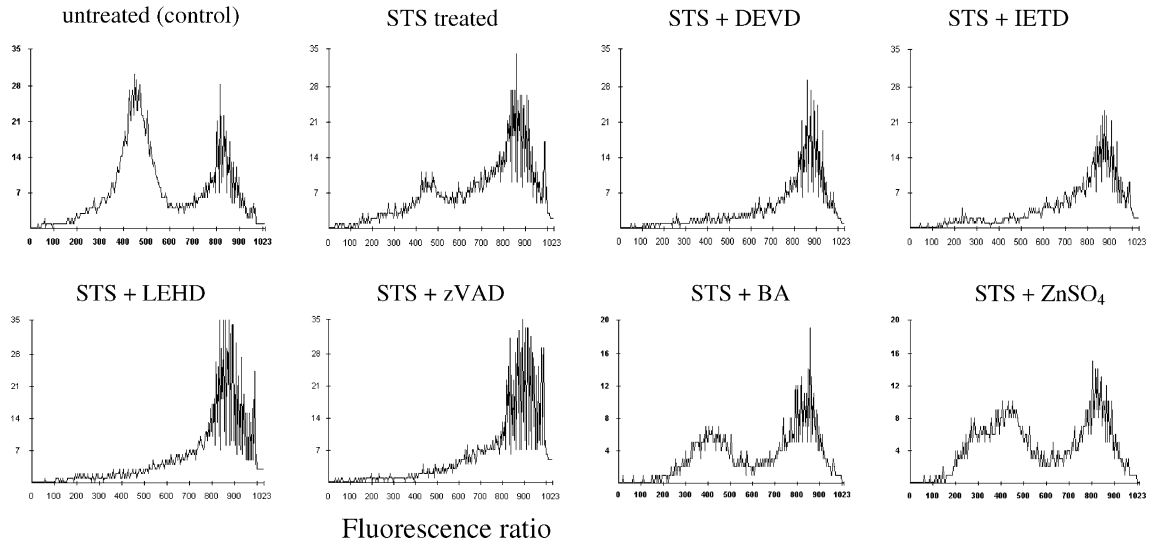

Figure 7. Affect of chemical inhibitors on HepZ mitochondrial depolarisation during STS-induced apoptosis. Inhibitor concentrations used were as detailed in Figure 6. Harvested cells were stained with JC-1 and analysed by flow cytometry by detecting fluorescence emission at 527 nm and 575 nm. J-ratio = 530 nm/590 nm.

by GSH peroxidase. Hepatic mitochondria have increased vulnerability to such effects as they lack the hydrogen peroxide metabolising enzyme, catalase. In contradiction to this hypothesis it was discovered that methionine did not induce the severe apoptotic response expected. A similar study in fibroblasts showed that depriving cultured fibroblasts of cystine caused GSH and thiol to fall to undetectable levels whilst deprivation of other sulphur-containing amino acids (e.g. methionine) had no marked affect on either GSH or thiol levels (Aoshiba et al. 1999).

The question of the mechanisms at play in the regulation of apoptosis by amino acids has been an

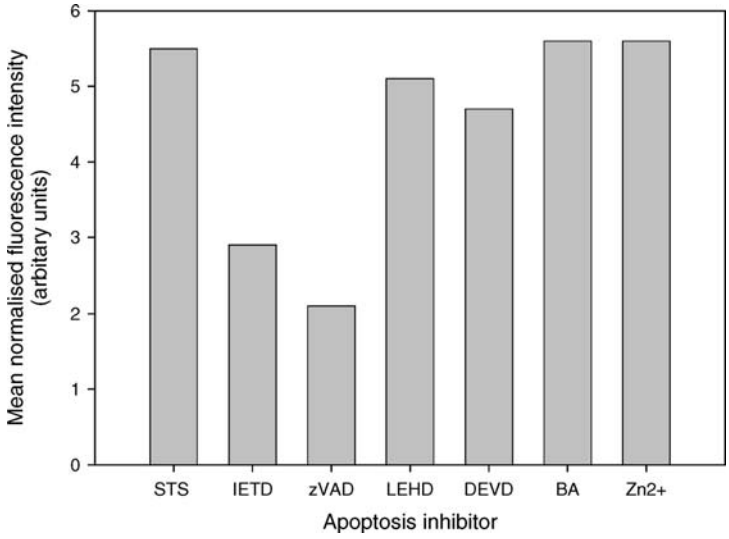

Figure 8. Influence of chemical inhibition on caspase activation following STS treatment. Inhibitor concentrations used were as detailed in Figure 6. Caspase activity in harvested cells was determined by measuring fluorescence emission at 530 nm using flow cytometry. Data shown is normalised against control (untreated) culture.

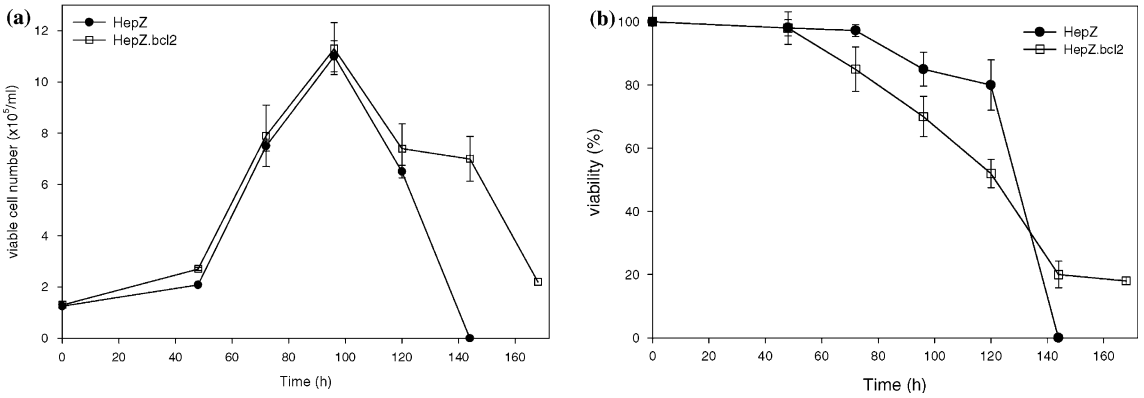

Figure 9. Role of Bcl-2 over-expression on cell growth (a) and viability (b) of HepZ in batch culture. HepZ at an initial density of 1.5×10^5 cells/ml were cultured in DMEM supplemented with 10% FCS in a humidified 5% air/95% CO_2 incubator at 37 °C. At each sampling point, the monolayer was trypisinised and resuspended in the spent medium and viable cell number determined by trypan blue exclusion. (a): Viable cell number. (b): Percentage viability. Data represents the mean ± S.E.M of thee independent experiments.

area of study that has ignited much research interest. Pathways proposed have implicated failure of nucleotide and protein synthesis, expression of 'new' pro-apoptotic genes, reduction of intracellular ATP pools due redox imbalance and collapse of mitochondrial membrane (Rabinovitz 1992; Mecille and Massie 1994; Galle et al. 1995; Xie and Wang 1996; Simpson et al. 1998). Clearly, amino acid deprivation can elicit an apoptotic response using several mechanisms.

Whilst the depletion of vital nutrient induces high levels of cell death, significant cell injury can result from accumulation of toxic metabolites during the late stages of the cell growth profile. Ammonia accumulation in particular, has been shown to inhibit cell growth (Dalili et al. 1989). However, in the present study no evidence of apoptosis was observed by fluorescence microscopy, in fact ammonia levels studied did not have a detrimental affect on either HepZ proliferation or survival. During HepZ batch culture, the cumulative ammonia levels recorded exceeded cytotoxic levels in hybridoma and myeloma cultures, with a concentration reaching 7.4 mM recorded (not shown) (Mecille and Massie 1994; Singh et al. 1994; Tey et al. 2000b). No adverse affects were observed on increasing ammonia levels to 14 mM, indicating that HepZ have retained the ability to metabolise excess ammonia *via* ureogenesis.

The crucial role of caspases in hepatocyte apoptosis is supported by a wealth of inhibition studies. Pre-treatment of hepatocytes with broad spectrum caspase inhibitor, z-VAD-FMK and more specific inhibition of caspase-3 (Ac-DEVD-CHO or z-DEVD-FMK), caspase-8 (z-IETD-FMK) and caspase-9 (z-LEHD-FMK) has provided evidence in the same way as had been reported in previously studied cell types and cell lines (Cain et al. 1996; Jones et al. 1998; Blom et al. 1999; Woo et al. 1999; Albright et al. 2003). Similarly, we show in the present study that staurosporine-induced apoptosis can be blocked by treatment with caspase inhibitors. However, the mechanism by which staurosporine induced apoptosis is apparently independent of the mitochondrial apoptotic pathway. It was suggested that multiple mechanisms may be involved in staurosporine-induced apoptosis and these may vary between different cell types (Zhang et al. 2004).

The observation of depolarised mitochondrial membranes in healthy, untreated cultures is not unique to HepZ and has been reported in human osteosarcoma cells where changes in $\Delta\psi_m$ have pro- and anti-apoptotic effects depending on the experimental model. In the latter, decreases in $\Delta\psi_m$ and thus a reduction in ATP for protein synthesis inhibited or delayed apoptosis until such time that reduced ATP levels were the cause of cellular demise (Salvioli et al. 2000). Human osteosarcoma cells were observed to undergo mitochondrial permeability transition and mitochondrial swelling in a reversible manner and with the marked absence of apoptosis (Minamikawa et al. 1999). Indeed the reversibility of the MPT may explain the heterogeneic response of HepZ mitochondria to STS treatment. Results

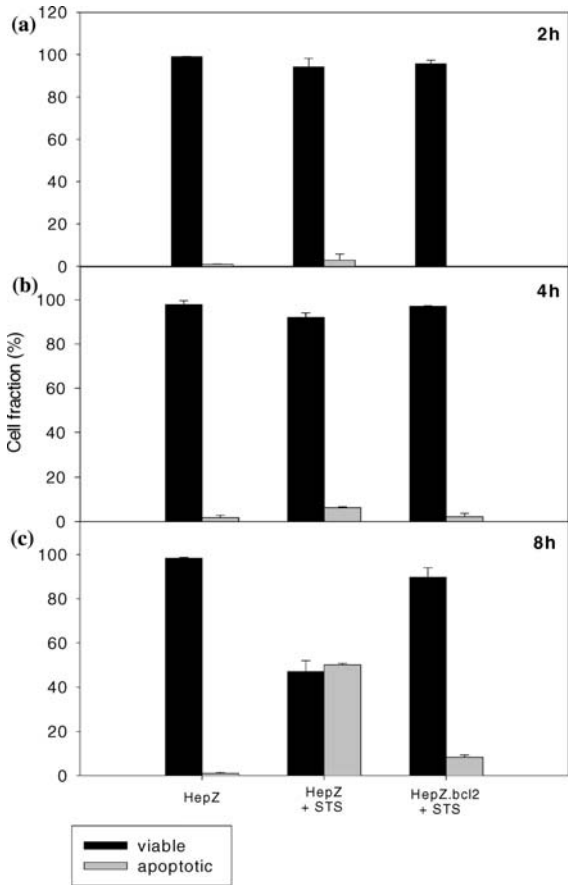

Figure 10. Protective effect of Bcl-2 over-expression on staurosporine (STS)-induced apoptosis. 5×10^5 cells were seeded into 6 well plates allowing triplicate wells to be sacrificed at each time point. Cells were incubated at 37 °C overnight before addition of staurosporine (2 μM). Apoptosis levels were assessed 2, 4 and 8 h after STS additions by AO/PI dual staining, cells were viewed by fluorescence microscopy (×200 magnification). HepZ and HepZ.bcl2 represent control cells and bcl-2 transfected cells, respectively. Data represents mean and ± S.D of three independent.

presented here suggest that collapse of mitochondrial membrane potential (MMP) is not necessarily a manifestation of cellular apoptosis but may in fact be a transitory or secondary event. A similar conclusion can be drawn from the chemical inhibition studies reported here where administration of caspase inhibitors drove mitochondrial depolarisation, even where STS-induced apoptosis was prevented. This raises uncertainty as to whether loss of mitochondrial membrane potential is a response to apoptotic stimuli (in this case STS) or merely a reflection of an ever-changing microenvironment. If this is so, the legitimacy of using mitochondrial depolarisation data, as has been done in the past, to infer apoptosis is debatable.

Since its discovery as a regulator of apoptosis, *bcl-2* has been seen as the prototype apoptosis suppressor gene and has been successfully over-expressed in many cell types including Burkitts lymphoma (Singh et al. 1996), hybridoma (Simpson et al. 1997, 1998, 1999; Ishaque and Al-Rubeai 2002), myeloma (Suzuki et al. 1997; Tey et al. 2000a), CHO (Fussenegger et al. 2000; Tey et al. 2000b) and hepatic cells (Lacronique et al. 1996). However, the assertion that over-expression of *bcl-2* is the answer to the apoptosis problem in biotechnology has come under increasing scrutiny. Reports that *bcl-2* exerts affect over mechanisms other than apoptosis such as cell cycle, cell proliferation and specific productivity have also brought its utility into question (Borner 1996; O'Reilly et al. 1996; Fussenger and Bailey 1998). Whilst at face value the assessment of the affect of *bcl-2* over-expression in HepZ yielded positive results *i.e.* reduction of $\Delta\psi_m$ under all the conditions studied and in the case of glucose- and cysteine-deprivation a drop in apoptosis, to fully understand the value of these findings they must be considered in the context of recent reports. In this study it is apparent that over-expressed *bcl-2* exerts its anti-apoptotic capacity by limiting mitochondrial membrane depolarisation but recent studies have suggested that the role of endogenously expressed *bcl-2* should not be overlooked (Murray et al. 1996). This is of particular significance in hepatic-derived cells, such as HepZ, that express elevated levels of endogenous Bcl-2. Endogenously expressed *bcl-2* (and *bcl-*x_L) can and often are up-regulated in the presence of apoptotic stimuli, often rendering any ectopically expressed bcl-2 redundant. Hence, questioning not only the attribution of cytoprotectivity to over-expressed *bcl-2 per se* but also the validity of introducing Bcl-2 expression plasmids into HepZ. Further still, increased endogenous *bcl-2* levels have been related to the selection strategies chosen, where neomycin and neomycin-based (*e.g.* puromycin) selection systems induce expression of endogenous *bcl-2*. Despite this, it is accepted that *bcl-2* is only protective against apoptosis at high expression levels. In these studies exogenous expression

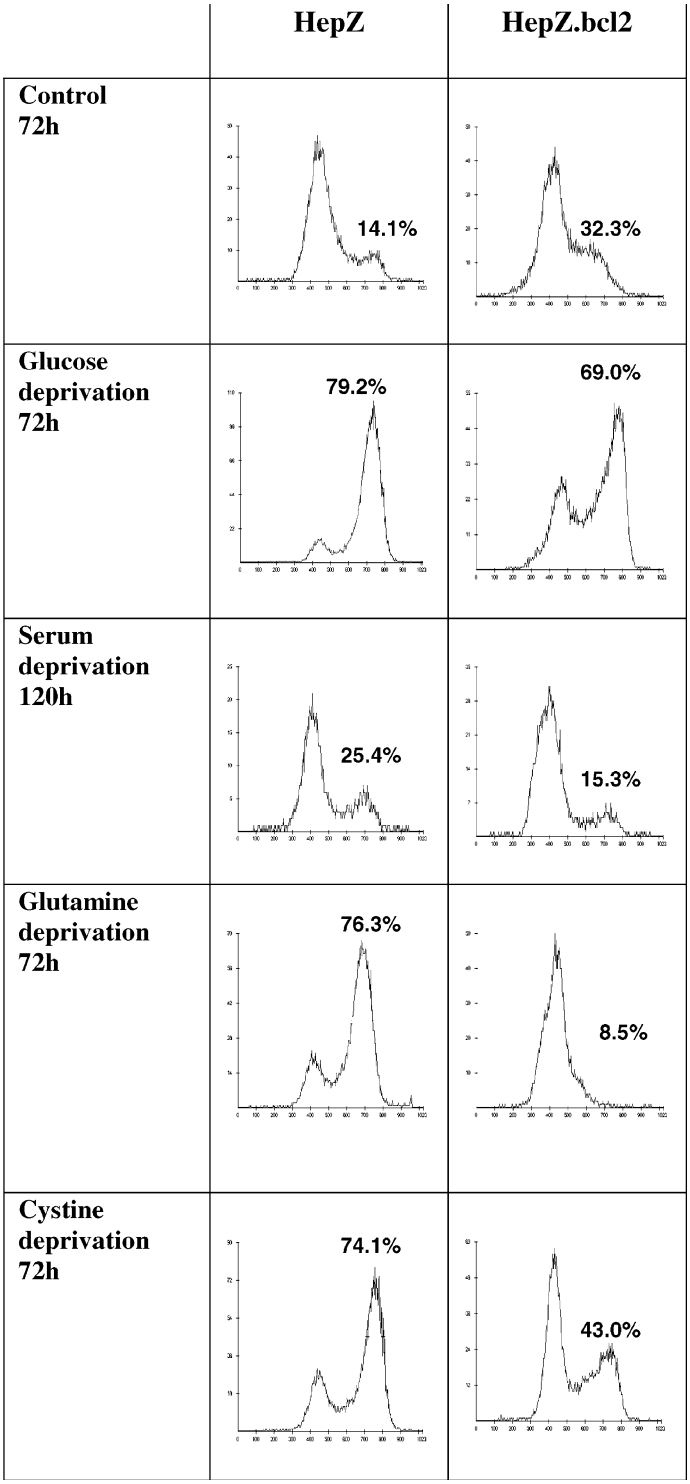

Figure 11. Mitochondrial membrane depolarisation in HepZ and HepZ.bcl2 upon nutrient deprivation. HepZ and HepZ.bcl2 were cultured in medium (DMEM + 10% FCS) that was deficient of glucose, serum, glutamine and cystine independently. Mean fluorescence ratio was determined by staining cells with JC-1 and flow cytometric detection of fluorescence emission at 530 nm and 590 nm.

of Bcl-2 protected cells from glutamine and glucose deprivation, serum depletion, chemical induced growth arrest, hypoxia and hyperoxia. A pioneering study by Lacronique et al. (1996) showed that the hepatocytes of transgenic mice expressing bcl-2 in their livers were protected from Fas-antibody mediated apoptosis. A later study has suggested that the role of Bcl-2 as a cytoprotective agent is dependent on the apoptotic stimuli (Chung et al. 1998). The exact mechanism(s) by which Bcl-2 elicits such widespread protection still elude researchers, although many authors have suggested Bcl-2 functionally compensates for absence of survival factors, in conditions of nutrient depletion. Others have suggested that Bcl-2 functions in an anti-oxidant fashion, where Bcl-2 over-expression correlates closely with increased GSH levels (Hockenberry et al. 1990).

Over-expression of Bcl-2 in HepZ reduced the cell vulnerability to some apoptotic stimuli and lengthened the batch culture. These findings are very much in agreement with many previous studies that showed that Bcl-2 only exerts its influence in sub-optimal culture conditions and not during the exponential growth phase (Singh et al. 1996; Simpson et al. 1997; Tey 2000a). Studies where apoptosis was induced by nutrient deprivation revealed that Bcl-2 protected against some stimuli and not others. In the absence of cystine and glucose, results strongly agree with previous findings (Singh et al. 1996; Simpson et al. 1997). In conditions of glutamine and serum deprivation, no protection is conferred suggesting that apoptosis under these conditions occurs *via* a pathway that is not associated with cytochrome *c* release and involvement of the Bcl-2 family. Similar conclusions have been drawn from studies of neuronal apoptosis due to serum withdrawal – where no evidence of either caspase activation or mitochondrial involvement was obtained (Hamabe et al. 2000; Colombaioni et al. 2002).

Conclusion

The apoptotic process in hepatocytes proceeds *via* both the death receptor-induced and mitochondrial-mediated pathways where the activation of caspases is key to apoptosis progression; in a mechanism that does not differ from that of other cell types. However, much like for other cell types, the true role (and significance) of mitochondrial depolarisation in hepatocytes apoptosis remains unresolved. Although *bcl-2* was found to be marginally cytoprotective to apoptosis induction in *in vitro* culture of hepatocytes, the results in this work brought to the fore the contradictions that surround the use of *bcl-2* over-expression as the archetypal anti-apoptotic strategy.

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