Special Issue

Apoptosis and its control in cell culture systems

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Abstract

Apoptosis is a form of programmed cell death which exhibits highly distinctive morphology. Research activity in this area has increased substantially in recent years, primarily due to the realisation that dysregulation of apoptosis is involved in the development of a number of pathological conditions, including cancer and AIDS. However, it is now clear that apoptosis also represents the dominant form of cell death during the culture of industrially important cell lines. This review focuses on the induction of apoptosis during industrial cell cultures as well as the effects of the apoptosis suppressor gene *bcl-2* on cell survival in conditions relevant to bioreaction environments. We also present new data which demonstrates that *bcl-2* can protect cells from apoptosis induced by oxygen deprivation, a finding which has important implications for large scale and intensive cultivation of cells. We also describe experiments which suggest that *bcl-2* can reduce the specific nutrient consumption rate of cells.

Introduction

The development of an embryo from a single cell to a fully formed individual requires not only cell proliferation, but also high levels of programmed cell death. Cells are produced in excess, and the embryo is effectively sculpted by the controlled elimination of particular cells at specific points in the developmental programme (Glucksman, 1951; Saunders, 1966; Farbman, 1968). Clearly, such a process has to be undertaken under tight genetic control, and must be clean and rapid, allowing for the disposal of the dead cell without causing damage to surrounding tissue. This contrasts markedly with accidental or necrotic death, in which large scale cell damage leads to the metabolic collapse of the cell. In such cases the cell becomes swollen and bursts, releasing its contents into surrounding tissue and causing inflammation therein.

The recognition that programmed cell death is not simply a feature of development, but continues to play a vital role in tissue kinetics in the fully formed adult has been largely responsible for the intense interest in the phenomenon in recent years. Indeed, all cells now appear to possess the capacity to undergo programmed cell death. This fundamental role is also reflected in

the growing evidence that links aberrant induction or failure of programmed cell death to the development of conditions such as cancer (Harrington *et al.*, 1994) and AIDS (Ameisen, 1992; Meysaard *et al.*, 1992; Laurent-Crawford *et al.*, 1991; Finkel *et al.*, 1995).

Consequently, few areas of cell biology remain untouched by apoptosis research, and studies over the last few years confirm that animal cell technology is no exception. Despite the levels of cell death seen during large scale culture of mammalian cells, study of the factors responsible has remained a surprisingly neglected area of research. Of those studies that have investigated the causes of cell death, all had initially assumed that death occurred by necrosis. However, it is now clear that, at least for some cell lines, death in bioreactors occurs by programmed cell death.

Morphology of apoptotic death

The studies of Kerr, Wyllie and Currie during the early 1970s are often cited as important milestones in the study of programmed cell death (Kerr *et al.*, 1972). They described a morphologically distinct form of programmed cell death and coined the now widely used term “apoptosis”, which is derived from the ancient

Figure 1. Transmission electron micrograph of an apoptotic cell showing condensation of chromatin.

greek word for the falling of leaves during autumn. Apoptotic cells undergo highly characteristic condensation and fragmentation of chromatin and cell shrinkage (Ohyama *et al.*, 1981; Thomas and Bell, 1981) (see Figure 1). They then undergo intensive blebbing, sometimes referred to as “boiling” of the plasma membrane, during which numerous protusions form at the surface of the cell. These protrusions eventually break-away from the plasma membrane, forming intact cytoplasmic packages called apoptotic bodies (Sanderson *et al.*, 1982). This process is believed to facilitate the phagocytosis of the dead cell by surrounding tissue and specialist macrophage. Thus, *in vivo*, all evidence of death is rapidly removed. *In vitro*, in the absence of macrophage, the apoptotic cell subsequently enters a degenerative phase during which it undergoes structural collapse, a phase which is often called secondary necrosis. The condensation of chromatin is accompanied by its nuclease mediated cleavage, initially into 300-750 Kb sized fragments, and eventually at the internucleosomal linker-regions, which generates μ l-timers of about 200 b.p. (Wyllie *et al.*, 1980; Oberhammer *et al.*, 1993). A further important biochemical feature of the process is the cross linkage of cytoplasmic proteins by an enzyme called tissue transglutaminase (Piacentini *et al.*, 1991). It is thought that this ensures that the dead cell is stabilised and does not burst prior to phagocytosis (Wyllie, 1993; Cohen, 1993).

Necrotic death, on the other hand, occurs in response to extreme environmental stress. Membrane damage leads to the failure of the cell to maintain ionic homeostasis, resulting in most cases in the swelling and eventually, bursting of the cell. Nuclear morphology does not undergo the condensation observed during

apoptosis, and the chromatin undergoes non-specific cleavage (for review see Trump *et al.*, 1981).

Apoptosis and biotechnology

There is a growing interest in the possibility that manipulation of the apoptotic pathway may provide new routes to the treatment of diseases such as cancer. Indeed, a number of companies are currently involved in the development of inducers or suppressers of apoptosis for application as therapeutic agents. For example, Hoechst Marion Rousel Inc. (Ohio, USA) are currently developing calpain inhibitors of apoptosis for the treatment of stroke victims. It would appear that much of the brain injury following ischemia is due to the induction of apoptosis in neurons, and the expression of bcl-2 (a proto-oncogene that inhibits apoptosis) using viral vectors can protect the cells from this form of death (Danheider, 1996).

In addition to the development of a range of novel therapeutic agents, apoptosis research may have a second, more fundamental, and perhaps more wide-ranging impact on animal cell technology. This stems from the recent demonstration that apoptosis is the primary mechanism of cell death during the culture of many industrially important mammalian cell lines. Thus the biochemical and/or genetic suppression of apoptosis may provide an additional route to the minimisation of death during the culture process.

One of the earliest indications that apoptosis may account for some cell death during the culture of murine B-cell hybridomas came from Al-Rubeai *et al.* (1990). Franek *et al.* (1991) made the first attempt to quantify the level of apoptosis during the death phase of a hybridoma. Based on the use of gel chromatography to measure DNA fragmentation, and on counting of apoptotic bodies, they suggested that around 30% of the cells at the end of batch culture were apoptotic. A number of groups have subsequently estimated levels of apoptosis during the death phase of murine plasmacytoma and hybridoma cell lines based on analysis of nuclear morphology (Singh *et al.*, 1994; Mercille and Massie, 1994a). They reported that as much as 90% of the dead cells during the death phase of these cultures were apoptotic. Solis-Recendez *et al.* (1995) used gel chromatography to establish the mode of cell death during the culture of a range of hybridoma cell lines. Out of 32 lines tested, only 8 failed to produce a ladder pattern, indicating that, of the cell lines screened, the majority underwent apoptotic death. The picture during CHO cultures is somewhat more confused. While Singh *et*

al. (1994) did not find conclusive evidence of apoptosis during serum-supplemented batch cultures of CHO cultures, Moore *et al.* (1995) have reported significant levels of apoptosis during serum-free cultures of CHO cells. In the light of these mixed results, further studies of CHO cells were initiated in our laboratory. These indicated that CHO cells may undergo apoptotic death although there was no evidence of blebbing or break-up of the cell into apoptotic bodies. Thus the failure to detect apoptotic cells may be due to the atypical and incomplete morphology exhibited by these cells. Alternatively, these cells may undergo significantly lower rates of apoptotic death, with more rapid progression into a secondary necrotic phase consequently making identification more difficult. Furthermore, there is the possibility that variability in sensitivity to apoptosis exists between different CHO cell lines, and different culture conditions may also affect the level of apoptosis. For example, cells cultured on serum-free media may be particularly sensitive to apoptosis.

Various strategies have been suggested to prevent apoptosis in cell culture (Cotter and Al-Rubeai, 1995). We report later in this article the recent investigations of the suppression of apoptosis by the over-expression of anti-apoptosis genes and their effects on cell viability under a range of culture conditions. Most of these studies have investigated the effect of the *bcl-2* gene. This proto-oncogene was identified at the t(14;18)(q32;q21) break point found in human follicular lymphoma (Tsu-jimoto *et al.*, 1985). The 24 KDa product of this gene is found to be associated with the outer mitochondrial membrane and the cytosolic face of the nuclear membrane and endoplasmic reticulum. Vaux *et al.* (1988) demonstrated that over-expression of this gene allows increased cell survival (without an increase in proliferation) following growth factor deprivation. Subsequent studies have demonstrated that this gene can also protect cells from apoptosis induced by numerous other agents. In addition to reviewing studies of the suppression of apoptosis during cell culture processes, we also present new data concerning the influence of *bcl-2* on cell survival under anoxic conditions and the effect of *bcl-2* on specific nutrient utilisation rates.

Materials and methods

Cell lines and cell maintenance

The Burkitt Lymphoma (BL) cell line MUTU-BL (derived from an EBV-genome positive tumour was

Table 1. Induction of apoptosis under cell culture conditions

Type of stress	Level of apoptosis
Serum deprivation	very high
Glucose deprivation	very high
Glutamine deprivation	very high
Low Oxygen	very high
High Oxygen	high
Moderate levels of hydrodynamic stress	moderate
High ammonia concentration	low
High levels of hydrodynamic stress	none

previously transfected by Milner *et al.* (1992) using *bcl-2* (pCΔ-*bcl-2*) and control (pCΔ-SV2) plasmids. Both control and transfectant cell lines were maintained in RPMI basal medium (Gibco BRL, UK) supplemented with 10% foetal calf serum (FCS; Gibco BRL, UK) in 50 and 200 ml T-flasks at 37 °C. Stability of *bcl-2* expression was monitored regularly by Western blot analysis.

Influence of *bcl-2* on amino acid utilisation

Suspension batch cultures of *bcl-2* transfectant and control cell lines were carried out in spinner-flasks. On day 3 of the culture, medium samples were removed from each culture and analysed for amino acid content using an HPLC system with cation exchange column (30 cm x 0.33 cm Aminex A8).

Influence of *bcl-2* on cell survival in anoxic conditions

Anoxic conditions were generated by sparging the culture medium alone with 10% carbon dioxide and 90% nitrogen for 15 minutes. This reduced oxygen to undetectable levels without affecting the pH. Cells taken from the mid exponential phase of batch cultures of the *bcl-2* transfectant and control cell lines were then transferred to the anoxic culture medium and anoxic conditions were maintained by surface aeration using 10% carbon dioxide and 90% nitrogen. Samples were removed at regular intervals over 35 h and viability and cell number were assessed using trypan blue exclusion and a haemocytometer respectively.

Results and discussion

One of the characteristic features of hybridoma cultures is the absence of a stationary phase, and an extreme-

Figure 2. a) Influence of bcl-2 on viable cell number during suspension culture of burkitt lymphoma cells. b) Specific amino acid and glucose uptake rates (between day 0–3) of the burkitt lymphoma suspension cultures.

ly rapid death phase. The onset of this death phase coincides with the exhaustion of glutamine. It should be of no surprise then that glutamine deprivation has been found to be one of the most important inducers of apoptosis. Additionally, deprivation of serum and glucose have both also been reported to be important inducers (Mercille and Massie, 1994a; Singh *et al.*, 1994; Franek, 1995). Table 1 summarises the level of apoptosis induced by each of the most important com-

ponents of the bioreactor environment. In a number of studies the possibility has been investigated that anti-apoptotic genes can protect cells from death following nutrient limitation, thereby extending the duration of batch cultures. Itoh *et al.* (1995) reported that bcl-2 had this effect on a murine hybridoma cell line, resulting in a 4 fold increase in antibody productivity. Simpson *et al.* (1996) found a similar enhancement in survival during the culture of a bcl-2 transfected hybridoma,

Figure 3. Influence of bcl-2 on cell number and viability following exposure to anoxic conditions.

but found only a 40% increase in antibody productivity. Singh *et al.* (1996) found that bcl-2 transfection of a burkitt lymphoma (BL) cell line enhances batch culture duration, and nutrient analysis revealed that bcl-2 over-expression allowed cells to survive following exhaustion of glutamine, with a significant fall in culture viability only occurring after subsequent exhaustion of glucose.

Figure 2a shows the changes in cell number during the suspension batch culture of both bcl-2 transfectant and control BL cell lines in suspension culture. These cells had never previously been grown in suspension culture, and consequently the control cell line died over the first 3 days of the suspension culture. However, bcl-2 over-expression clearly protected the cells from death, allowing cell division to take place. The levels of glucose and amino acid utilisation during batch cultures of the bcl-2 transfectant and the control cell lines are shown in Figure 2b. For all amino acids and glucose utilised, bcl-2 reduced the specific utilisation rate. Whether this is completely attributable to the suppression of death, or whether bcl-2 is able to modify the metabolic activity of viable cells so as to improve the efficiency of nutrient utilisation remains to be seen. It is possible that by conferring resistance to environmental insults, bcl-2 allowed the cells to minimise the specific utilisation rate of biosynthetic intermediates used for maintenance and repair process.

Oxygen limitation also plays a critical role in determining the maximum cell density of a culture. Studies by Mercille and Massie (1994b) demonstrated that oxygen deprivation could result in the induction of apoptosis. Figure 3 shows the effect of bcl-2 over-expression on cell death under anoxic conditions. Whereas the control cell line viability collapsed within the first 15 h of the experiment, the bcl-2 transfectant cell line exhibited a much lower rate of loss of viability over the first 30 h. However, within the next 5 h, there was a rapid collapse in viable cell number. Clearly, bcl-2 had protected the cells from cell death caused by anoxic conditions. Recent studies in our laboratory have demonstrated that bcl-2 can also protect murine hybridoma cells from anoxia induced apoptosis (Simpson *et al.*, 1996). Clearly these results have important consequences for large scale and intensive culture processes, during which oxygen limitations are common.

The significance of apoptosis research

The studies described above have important consequences for the production of biopharmaceuticals from mammalian cell lines. Firstly, they would indicate that increasing the duration of batch cultures leads to an increase in antibody productivity. Maintaining viability during the death phase may also be desirable from a downstream processing viewpoint, as it minimises the level of cell debris which has to be removed from the culture medium. Additionally, many of the intensive culture systems used for the cultivation of mammalian cell lines exhibit high levels of cell death, partly because of nutrient limitation. Suppression of apoptosis by the use of genes such as bcl-2 should therefore allow cell survival under these conditions, thus minimising the proportion of culture resources which are required to generate replacement biomass. As with batch culture, this would also reduce the complexity of the culture medium, thereby simplifying the recovery of the expressed protein.

The over-expression of bcl-2 can also protect cells from apoptosis induced by serum deprivation. Previously, it was thought that serum provided the cell primarily with factors required to stimulate proliferation. However, demonstration of the role of serum-derived survival factors (for review see Raff *et al.*, 1993) in protecting cells from apoptosis has important implications for the formulation of new, more effective and perhaps simpler serum-free media. Studies by Singh *et al.* (1996) have demonstrated that

bcl-2 over-expression enhances survival in commercially available serum-free media, leading to improvements in the maximum cell number of the culture. Studies are currently underway to establish the effects of a variety of anti-apoptotic factors on cell death following serum withdrawal. The objective is to include these chemicals in a novel serum-free medium formulation which is designed to suppress apoptosis, in addition to agents which stimulate cellular proliferation.

In previous work, strategies for optimising specific protein productivity have been proposed based on restriction of the cell proliferation rate, for example, through the use of thymidine to arrest cell growth (Al-Rubeai *et al.*, 1992). However, subsequently, Singh *et al.* (1994) have demonstrated clearly that such strategies result in the induction of apoptosis and any production enhancing effects are of limited duration. However, studies in our laboratory have demonstrated that over-expression of bcl-2 in a burkitt lymphoma (Singh *et al.*, 1996) and a murine hybridoma (Simpson *et al.*, 1996) allows cell death to be delayed for many days following thymidine treatment. Future studies which attempt to use control of growth rate in order to stimulate protein productivity must therefore take into account the induction of apoptosis, and include strategies specifically designed to minimise this form of cell death.

Future prospects

Many questions regarding the work described above remain to be answered. For example, are there apoptosis-resistant hybridoma cell lines, and is there a correlation between resistance to apoptosis and general robustness? If there is such a correlation, it may be possible to use susceptibility to apoptosis as a simple predictor of cell robustness, thus aiding the process of screening cell lines for use in production processes.

A further question which arises from recent studies is; how does suppression of apoptosis lead to an improvement in product productivity? Does this reflect increased specific productivity, or increased numbers of cell factories due to the maintenance of culture viability? Also, what is the molecular fidelity of the product molecule released during the "apoptosis suppressed" phase of the culture?

It is generally accepted that the level of protection afforded by genes such as bcl-2 is dependent upon the level of expression of this protein. Consequently, we would expect that such high levels of bcl-2 expression would have an effect on the level of expressed protein

productivity. Indeed, during the exponential growth phase of a batch culture, the metabolic load placed upon the cell by cell-expression does reduce the rate of cell growth under the ideal conditions which prevail. Consequently, it may be necessary to screen other more potent suppressors of apoptosis which can provide the same degree of protection at lower levels of expression. Perhaps expression of ICE anti-sense of ICE-family proteins which are able to suppress apoptosis would achieve complete inhibition of death at the lowest level of expression of the suppresser protein. This should ensure that there is no negative effect on growth rate and productivity stemming from the synthesis of large quantities of the apoptosis-suppressing gene.

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