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Bcl-2 Over-expression Reduced the Serum Dependency and Improved the Nutrient Metabolism in a NS0 Cells Culture

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Abstract The over-expression of Bcl-2 has greatly improved the culture period, specific growth rate, and maximum viable cell density of NS0 cells culture under low serum condition. Further analysis of these data suggests that a saturation model of the Monod type can be used to represent the relationships of specific growth rate and initial serum concentration. The μ_{max} and K_s for the Bcl-2 cell line is 0.927 day^{-1} and 0.947% (v/v) respectively, which are 21% greater and 7% lower respectively than its control counterpart. Study on the amino acid supplementation revealed that Bcl-2 cell lines possess greater improvement in the specific growth rate and maximum viable cell density compared to the control cell lines. A further increase in the amino acid supplementation has resulted a 17% decrease in specific growth rate and no improvement in maximum viable cell density in the control culture. However, the Bcl-2 cell line exhibited a better growth characteristic in this culture condition compared to that of control cell lines. The higher specific growth rate and maximum viable cell density of the Bcl-2 cell line in medium fortified with serum and MEM EAA suggested a more efficient nutrient metabolism compared to that in the control cell line. The low serum and amino acid utilisation rate and the higher cell yield may prove to be important in the development of serum/protein free culture.

Keywords: apoptosis, *bcl-2*, NS0 cells, serum, essential amino acid, specific growth rate

INTRODUCTION

Animal cells are the preferred "cell factories" for the production of biopharmaceutical used in therapeutics or diagnostics. These complex molecules required the correct post-translational processing (including glycosylation), which is absence in prokaryotic microbial cells. This post-translational modification in many cases is essential for full biological activity of these products. However, there are several limitations with animal cell cultures compare to its microbial counterpart. The nutritional requirements of animal cells are complex. They require complete mixtures of minerals, vitamins and almost all amino acids. Besides that, they usually require specific growth factors, which are often added in the form of serum, or otherwise animal originated protein and lipid components. Serum may contain infectious agents like viruses and prions. Therefore, the use of serum is objectionable from a regulatory standpoint. Beside that, serum is expensive and has high protein content. Hence, serum removal would minimize down-stream processing requirements and reduce medium cost. Due to these reasons, the serum-free and protein-free culture media are in high demand. Several approaches have been used to eliminate the requirement for serum from animal cell cultures. Early attempts are focus on the selection of cell variants that would grow, in the absence of serum. Some animal cells have also been genetically modified to over-express specific proteins such as cyclin-E, thus allowed cell proliferation in serum and protein free medium [1]. The identification and purification of non-animal originated biologically active peptides and proteins have been an important and necessary step in allowing the development of serum free medium to mature. These non-animal originated components are used as supplement to replace serum during medium formulation.

The development of serum and protein free mediums can be considered very successful. Serum and protein free mediums are widely used in the commercial cultivation of animal cells. However, the apoptotic death rate in serum or protein free culture is usually higher than the serum supplemented culture. Zanghi *et al.* [2] showed that addition of serum to CHO cells grown in protein free medium could substantially reduce apoptosis induced by nutrient deprivation. Modulation of the apoptotic pathway by the over-expression of the *bcl-2* gene provides highly effective approach to the reduction of apoptosis in cell culture [3]. This approach has been demonstrated to be very successful for several industrially important cell lines including hybridoma [4,5], myeloma NS0 [6] and

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CHO cells [7-9]. Hence, one of the objectives of the present study is to investigate the ability of Bcl-2 protein on the reduction of serum dependency and the prevention of apoptosis induced by serum deprivation in NS0 cell culture.

Nutrient limitation is one of the primary causes of cell death during the decline phase of batch culture. The role of nutrient limitation in the induction of apoptosis in industrially important animal cell lines has been extensively studied [3]. Initially studies were restricted to the roles of glucose, glutamine, cysteine, and methionine [10,11]. However, recent studies suggest that cell death proceeds by apoptosis following the deprivation of any amino acid, with the effect being more significant following deprivation of essential amino acids [12]. In our previous studies, the effect of the limitation of several nutrients on the induction of apoptosis in NS0 and CHO cell lines was examined [6,9]. Among these nutrients, deprivation of amino acids was the most potent apoptosis inducer, followed by glucose, serum, and the substitutes for glutamine (glutamate and asparagine). In both cell lines, glutamine is synthesised from glutamate and ammonia by glutamine synthetase (GS) and its metabolic requirement is also reduced with the addition of elevated levels of asparagine, whose biosynthesis is dependent upon glutamine [13].

The GMEM media used in this study has relatively low amino acid concentrations compared to other media such as DMEM. Results from the nutrient deprivation experiments and fed batch culture of our previous study have revealed the importance of amino acid feeding for enhanced cell growth and productivity [6]. Therefore, the influence of Bcl-2 over-expression on nutrient metabolism and cell growth was monitored in the present study with the objective of identifying the optimal amino acid concentration, which can support both higher growth rate and viable cell density.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The parent cell line NS0 6A1 was kindly supplied by LONZA Biologics (Slough, UK) and had previously been transfected with the glutamine synthetase (GS) expression system carrying a gene for a human-mouse chimeric antibody (cB72.3). The cells were transfected either with the *bcl-2* gene (NS0 Bcl-2) or the negative transfection vector, which gives the control cell line (NS0 Control), as previously described [6]. The NS0 Bcl-2 cell line over-expresses the 'anti-apoptotic' human Bcl-2 protein. Cells were maintained in GMEM medium (Gibco, Paisely, UK) supplemented with 7.5% foetal calf serum (FCS) (Gibco, Paisley, UK), MEM non-essential amino acids (Gibco, Paisley, UK), 1 mM sodium pyruvate (Gibco, Paisely, UK), 500 μM glutamic acid, 500 μM asparagine, 30 μM adenosine, 30 μM guanosine, 30 μM cytidine, 30 μM uridine, 10 μM thymidine, 2.7 g/L sodium bicarbonate, and 100 μM methylionine sulphoximine (MSX) (all chemicals from Sigma, Poole, UK).

Cell Count

Cell concentration was determined by haemocytometer count (improved Neubauer–Haemocytometer), and the trypan blue exclusion method was used to distinguish viable from non-viable cells.

Fluorescence Microscopy: Acridine Orange–propidium Iodide Staining

This method was used in addition to the trypan blue staining method in order to determine the distributions of viable, early apoptotic, late apoptotic, necrotic, and ghost cells. Each cell suspension sample was mixed with an equal volume of staining solution containing 10 mg/mL acridine orange (AO) and 10 mg/mL propidium iodide (PI). The mixture was loaded into an improved Neubauer rhodium-coated haemocytometer and cell numbers determined under fluorescence microscopy (excitation by UV light). By examination of colour and chromatin morphology the cells were classified and the results expressed as percentages of the total cells. Cells that exclude PI appeared green. If such cells showed a diffuse chromatin, they were counted as viable cells. Otherwise, if their chromatin was condensed, they were scored as early apoptotic cells. Red cells included PI and were counted as late apoptotic if their chromatin was condensed or as necrotic if they showed a diffuse chromatin. Chromatin free cells were counted as ghost [6].

Serum Limitation and Amino Acid Deprivation

Stock cultures of NS0 Bcl-2 and control cell lines were established in 250 cm^3 Spinner at an initial cell concentration of 2×10^5 cells/mL. Cells were removed at mid exponential phase and resuspended at 2×10^5 cells/mL in GMEM medium (Gibco, Paisley, UK) which was deficient in all amino acids for serum deprivation experiment. Serum deprivation experiments were carried out at a concentration of 0.5% FCS. The cell suspension was transferred in 10 mL aliquots to triplicate 25-cm^2 T-flasks. The cultures were incubated at 37°C in a humidified CO_2 incubator. On day 3, the cells were harvested and analysed as described above.

Batch Culture in Medium Containing Increased Initial Essential Amino Acids (EAA) Concentration

Stock cultures of NS0 Bcl-2 and control cell lines were established in 250 cm^3 Spinner at an initial cell concentration of 2×10^5 cells/mL. At mid-exponential phase of the culture, cells were harvested by centrifugation and resuspended at 2×10^5 cells/mL in fresh culture medium as described above and supplemented with 0×, 0.5× and 1× concentration of MEM EAA mixture and transferred into triplicate 25-cm^2 T-flasks. The cultures were incubated at 37°C. Samples were taken daily in order to assess viable cell concentration and viability as described above.

Batch Culture in Medium Containing Increased Serum Concentration

Stock cultures of NS0 Bcl-2 and control cell lines were established in 250 cm^3 Spinner at an initial cell concentration of 2×10^5 cells/mL. At mid-exponential phase of the culture, cells were harvested by centrifugation and resuspended at 2×10^5 cells/mL in fresh culture medium as described above and supplemented with 1%, 2.5%, 5%, and 10% FBS and transferred into duplicate 100-mL spinner flask. The cultures were incubated at 37°C Samples were taken daily in order to assess viable cell concentration and viability as described above.

Calculation of Specific Growth Rates

A specific growth rate, μ, is calculated using the following equations: In a batch culture,

$$\frac{d(x_v)}{dt} = \mu \cdot x_v - k_d \cdot x_v \quad (1)$$

During the exponential phase, the death rate constant, $k_d = 0$, and therefore the rate of dead cell accumulation are negligible and this expression reduces to:

$$\mu \approx \frac{1}{x_v} \cdot \frac{dx_v}{dt} \quad (day^{-1}) \quad (2)$$

or, by integration, $$\mu = \frac{\ln x_{v2} - \ln x_{v1}}{t_2 - t_1} \quad (3)$$

where x_{v2} and x_{v1} are the concentration of viable cells at time t_2 and t_1, respectively. The error in measuring μ ($\Delta\mu$) between these two points can be calculated from the error combination formulae [14]. The final equation is as follows:

$$\left|\frac{\Delta\mu}{\mu}\right| = \left|\frac{\Delta x_{v2}/x_{v2}}{\ln\frac{x_{v2}}{x_{v1}}}\right| + \left|\frac{\Delta x_{v1}/x_{v1}}{\ln\frac{x_{v2}}{x_{v1}}}\right| \quad (4)$$

where Δx_{v1} and Δx_{v2} are the standard error of x_{v1} and x_{v2}, respectively.

RESULTS AND DISCUSSION

Batch Culture in Medium Containing Increased Initial Serum Concentration

The control cells cultured in the medium supplemented with 1% serum were unable to proliferate and survive no longer than 4 days in batch culture (Fig. 1). The initial specific growth rate and maximum viable cell density of the control cell line increased with increasing serum concentration as shown in Fig. 2. The initial specific growth

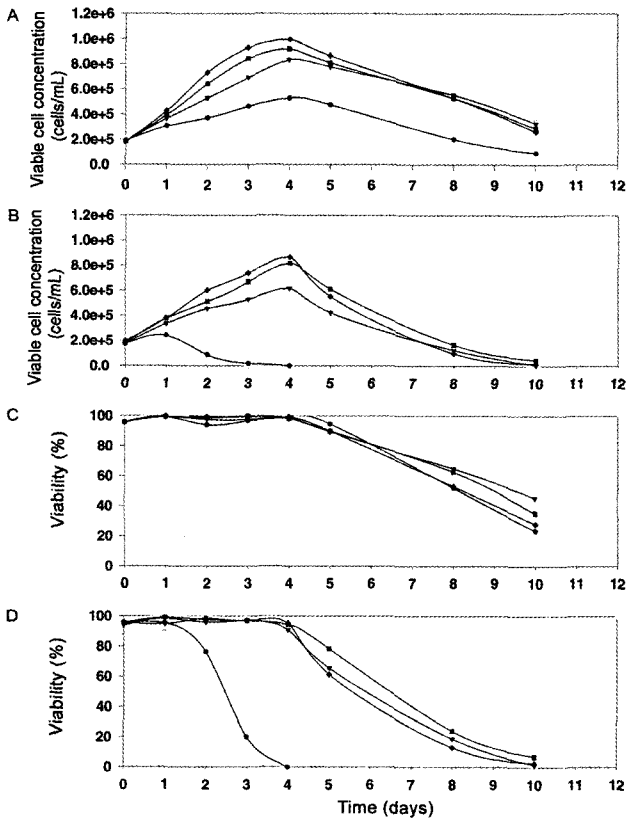

Fig. 1. Batch cultures of NS0 6A1 cells in medium containing increased initial serum (FBS) concentration: A, Viable cell concentration of Bcl-2, B, Viable cell concentration of control, C, Viability of Bcl-2, and D, Viability of control(Keys: circle; 1%, inverted triangles; 2.5%, square; 5% and diamond; 7.5%).

rate and maximum cell density of Bcl-2 cultures were highest at serum concentration of 7.5%, which were 0.630 day^{-1} and 8.66×10^5 cells/mL, respectively. The maximum viable cell concentration and specific growth rate of the Bcl-2 cell line in media supplemented with 1% serum were 120% and 40% higher than that of the control cell line. The viability of this culture remained above 20% after 10 days in batch culture (Fig. 1). The maximum viable cell density and specific growth rate of the Bcl-2 culture also increased with an increase in serum concentration. The highest specific growth rate and maximum viable cell density were found in the 7.5% serum culture and were respectively 25% and 15% higher than the control cell line. The viability of the Bcl-2 culture in other serum concentrations ranged from 25% to 50% at day 10 of batch culture, while the control cell line had completely lost viability in the same period of time.

The Monod model can be used to descript the dependency of NS0 cells on serum. The Monod model:

$$\mu = \frac{\mu_{max} \times S_o}{K_s + S_o}$$

where S_o is the initial substrate concentration, has been used to describe the effect of serum concentration on initial specific growth rate by Dalili and Ollis [15] and Lee-

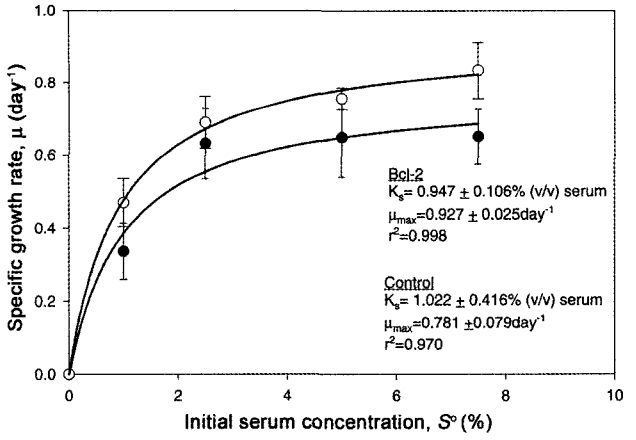

Fig. 2. The dependency of specific growth rate, μ, of NS0 6A1 cells on initial serum concentration, S°, (%) (Keys: open circle - Bcl-2, closed circle - control).

lavatcharamas *et al.* [16]. The values for the maximum specific growth rate, μ_{max} and Monod constant K_s were obtained *via* the SigmaPlot regression program. The μ_{max} and K_s for the Bcl-2 cell line is 0.927 day^{-1} and 0.947% (v/v), which are 21% greater and 7% lower respectively than the corresponding figures for the control cell line as shown in Fig. 2. The lower K_s value for the Bcl-2 cell lines means that it requires less serum compared to the control cell lines.

Our present results clearly demonstrated that over-expression of Bcl-2 protein has a positive effect on apoptosis regulation. Bcl-2 cell lines are able to grow and survive under low serum concentration should eventually lead to the development of more simple serum-free media or at least allow rapid adaptation of the cells to protein-free conditions. Indeed, over-expression of Bcl-2 was demonstrated to lead to an improvement in the growth of plasmacytoma cells in commercially available serum-free media designed for hybridoma cell lines, without the need for prior adaptation [17]. Fassnacht *et al.* [18] has also reported that a *bcl-2* transfected hybridoma cell line grew well in an iron-rich protein-free medium after 3 weeks adaptation compared to over 2 months taken by the control cell line.

Batch Culture in Medium Containing Increased Initial EAA Concentration

A simple experiment was carried out to investigate the influence of initial amino acid concentration on cell growth, with the objective of identifying the optimal amino acid concentration, which can support both higher growth rate and viable cell density. The results of the experiment are summarised in Figs. 3 and 4. The specific growth rate for control and Bcl-2 cell lines were 0.5% and 17% higher respectively in the media supplemented with 0.5× MEM EAA when compared with ordinary GMEM media. However, the maximum viable cell density was increased by 47% in Bcl-2 cell line compared to only 37% increased in

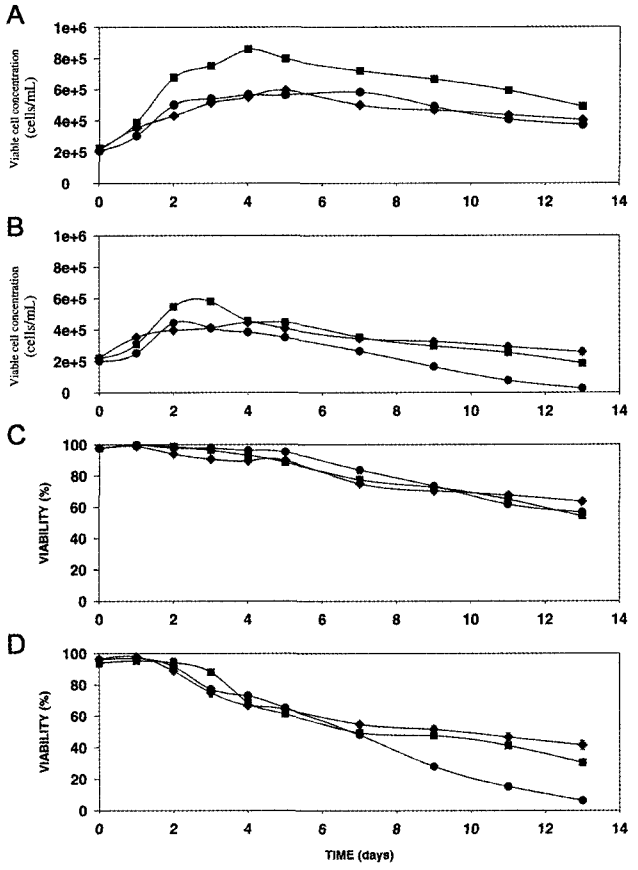

Fig. 3. Batch culture of NS0 6A1 cells in medium containing increased initial MEM essential amino acids (EAA) concentration and supplemented with 5% serum under static culture conditions: A, Viable cell concentration of Bcl-2, and B, Viable cell concentration of control, C, Viability of Bcl-2, D, Viability of control (Keys: circle; GMEM, square; GMEM + 0.5× MEM EAA and diamond; GMEM + 1× MEM EAA).

control cell line. These results show that the improvement in maximum viable cell density was not due to the increase in growth rate but was mainly the result of the delay in cell death by the higher level of amino acids, which allowed the accumulation of viable cells. A further increase in the amino acid supplementation from 0.5× to 1× resulted has caused a 17% decrease in specific growth rate and no improvement in maximum viable cell density in the control culture. However, the Bcl-2 cell line exhibited a better growth characteristics in media supplemented with 1× MEM EAA compared to its control counterpart.

It has been reported that fortification of the basal medium by balanced mixtures of amino acids and vitamins was more effective than daily supplementation of glucose and glutamine in improving culture viability and antibody productivity in protein free hybridoma batch cultures [19]. Supplementation with amino acid mixtures was also found to prevent starvation-induced apoptosis in hybridoma cells [20]. In the present study, NS0 cells cultured in a medium fortified with 0.5× MEM EAA mixtures exhibited

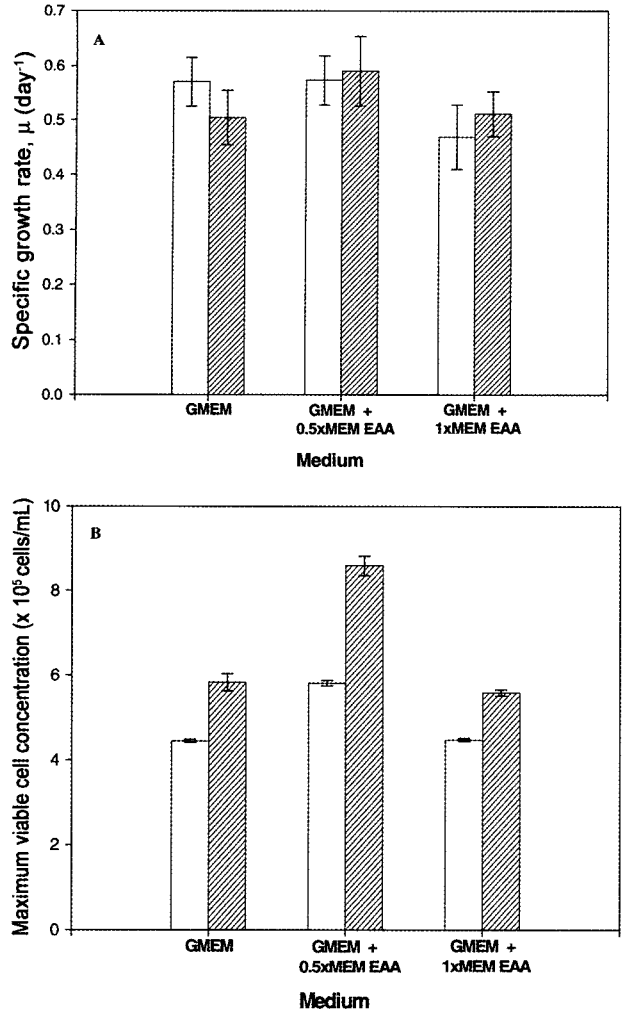

Fig. 4. A, Specific growth rate and B, Maximum viable cell concentration of NS0 6A1 cell line batch culture in GMEM medium containing increased initial MEM essential amino acids (EAA) concentrations and supplemented with 5% serum under static culture conditions (Keys: open bars; control, hashed bars; Bcl-2).

a substantially increased maximum viable cell density and specific growth rate as well as improved culture viability in the control cell line during the decline phase of batch cultures. However, a further increase in the EAA concentration to 1× has reduced the specific growth rate and did not bring any additional benefit in terms of maximum viable cell density compared to the unfortified medium, although it did improve the culture viability of control cells during the decline phase of batch cultures (Figs. 3 and 4).

The presence of 0.5× EAA significantly reduced cell death in the control cell line, which led to an increase in maximum viable cell density but the specific growth rate was not significantly increased. Conversely, the specific growth rate of the Bcl-2 cell line increased by 17% in the medium fortified 0.5× EAA, which led to a 47% increase in maximum viable cell density compared to a 31% increase in the control cell line. The higher specific growth rate and maximum viable cell density of the Bcl-2 cell line in medium fortified with EAA suggested a more efficient nutrient metabolism compared to that in the control cell line. Indeed, Simpson *et al.* [12] has reported that *bcl-2* transfected hybridoma cells were more efficient at utilising amino acid than the control cells. This finding together with that of Ishaqe and Al-Rubeai [21,22] indicates a higher efficiency utilization of nutrients by the Bcl-2 cells. Clearly, this characteristic is very important in high cell density perfusion cultures. Lower nutrient levels are required by the Bcl-2 cell line in order to obtain a similar amount of cellular biomass. Indeed in high cell density perfusion culture, over-expression Bcl-2 has allowed the same NS0 cell line to survive and proliferate to achieve a higher cell density without the need for additional nutrient compared to the control culture [23].

Nutrient Deprivation linduced Apoptosis in NS0 Cell Culture

The death mechanism of NS0 cells under serum and amino acid deprivation was assessed by exposing the cells to GMEM medium containing only 0.5% serum, and deficient of all amino acids. Cells were subjected to these conditions for a period of three days following initial inoculation of 2×10^5 cells/mL. The final viable cell density in the Bcl-2 cells was significantly higher than that of the control cells. Fluorescence microscopic data shown in Tables 1 and 2 confirmed that cell death had preceded predominantly by apoptosis in the control cultures. The majority of the dead cells in the control cultures were categorised as late apoptotic and ghost, which represents cells in the terminal stage of apoptosis. In the culture of Bcl-2 cell line, substantial of the dead cells exhibited necrotic morphology; again indicating that Bcl-2 overexpression had been highly effective at completely suppressing apoptosis in these cells. The presence of large number of necrotic cells in serum limitation Bcl-2 culture may probably due to the absence of other serum components such as attachment protein, lipids, and albumin (which may provide protection against shear stress induce by the suspension culture).

The mechanism of Bcl-2 protein prevents apoptosis upon growth factor withdrawal is by blocking the release of mitochondrial inter-membrane space pro-apoptotic molecules, such as cytochrome c and apoptosis inducing factor (AIF) [24]. Cytochrome c is required for the activation of caspases, a group of proteolytic enzymes involved in the apoptotic process. The AIF released from mitochondria accumulates in the nucleus. Inside the nucleus, AIF causes partial chromatin condensation in the periphery of the nucleus and the degradation of DNA into fragments greater than 50 kb in length [25].

In a review, Al-Rubeai [26] suggested that apoptosis is initiated when the maintenance energy required to produce ATP to drive the biosynthesis of cellular material is reduced to a critical level due to nutrient deprivation, resulting in the activation of a regulatory protein that pro-

Table 1. Composition of different cell type of NS0 cells after 3 days cultivation in GMEM medium supplemented with 0.5% serum

	* Viable cell concentration ($\times 10^5$ cells/mL)	Cell type (%)				
		Viable	Early apoptotic	Late apoptotic	Necrotic	Ghost
Control	0.031 ± 0.007	55.4 ± 0.2	1.6 ± 0.2	16.8 ± 0.5	7.4 ± 0.3	18.8 ± 0.2
Bcl-2	1.50 ± 0.01	85.5 ± 0.5	0.5 ± 0.1	1.0 ± 0.3	12.0 ± 0.5	1.0 ± 0.4

* Initial inoculation was 2.00×10^5 cells/mL.

Table 2. Composition of different cell type of NS0 cells after 3 days cultivation in GMEM medium deficient of all amino acids

	* Viable cell concentration ($\times 10^5$ cells/mL)	Cell Type (%)				
		Viable	Early apoptotic	Late apoptotic	Necrotic	Ghost
Control	0.031 ± 0.007	0.5 ± 0.1	0.7 ± 0.1	16.7 ± 0.3	6.9 ± 0.1	75.2 ± 0.4
Bcl-2	1.50 ± 0.01	64.0 ± 0.5	1.9 ± 0.1	9.8 ± 0.8	10.1 ± 0.5	14.2 ± 0.8

* Initial inoculation was 2.00×10^5 cells/mL.

motes apoptosis. The multiple roles of amino acids in cellular biosynthesis, metabolism, and regulation contribute to the fact that their deprivation leads to very high levels of apoptosis. Clearly, the deprivation of amino acids inhibits protein biosynthesis. Indeed, Mercille and Massie [11] have demonstrated that inhibition of protein biosynthesis by cycloheximide treatment results in the induction of apoptosis in hybridoma cells. This would indicate that the proteins required for the induction of cell death are pre-synthesised, but are kept in check by a regulatory protein with a short half-life. Failure of protein biosynthesis, especially the 'apoptotic regulatory protein', consequently condemns the cell to death. Amino acids can also contribute to the cellular energy requirement, which is also essential for protein biosynthesis. Xie and Wang [27] estimate the energy contributed by amino acids to be around 11%, with leucine, isoleucine, threonine, and valine being the most important. Rabinovitz [28] hypothesised a general mechanism that the general demise of metabolism (the 'pleiotypic response') in response to amino acid deprivation is the result of interactions between components of the glycolytic and protein synthesis pathways. Accumulation of uncharged tRNA leads to the inhibition of phospho-fructokinase (PFK), the levels of glyceraldehyde-3-phosphate decline, which ultimately leads to the reduction of phospho-ribosyl-pyro-phosphate, a step that introduces ribose into the synthesis of purine and pyrimidines. With this mechanism, deprivation of amino acid would lead to the inhibition of nucleotide synthesis. The insufficient supply of nucleotides would lead to cell cycle arrest and consequently induction of apoptosis [29]. A study conducted by Simpson *et al.* [12] showed that apoptosis was induced following the deprivation of any single amino acid individually. The deprivation of EAA exhibited a greater loss of viability and higher degree of apoptosis than the deprivation of non EAA (NEAA). This is not surprising since the cell itself can synthesize the NEAA; while the supply of EAA is solely depend on the medium supplementation.

Apart from protein synthesis, metabolic energy, and DNA synthesis, amino acid homeostasis has been functionally implicated in: hormone metabolism, catalytic functions, regulation of cell growth, and nitrogen metabolism [30]. Indeed, the studies of Franek and Sramkova [31-33] involved the systematic amino acid starvation of hybridoma cells, which led to the identification of a set of 'signalling' amino acids. These 'signalling' amino acids could suppress apoptosis in the starvation medium even if present at low concentrations. Thiol group amino acids such as L-cysteine and L-tryptophan have been reported to prevent apoptosis induced by serum depletion and oxidative stress [34,35]. Interestingly, other thiol-bearing and dithiol-cleaving compounds exhibited a similar ability to rescue the cells whereas non thiol-containing reducing agents (*e.g.* superoxide dismutase) were ineffective in apoptosis inhibition [34].

In the present study, when cells were deprived of all amino acids simultaneously, the accumulation of 'ghosts' in the NS0 cultures indicated that cell death was by apoptosis. One may have expected that complete absence of all amino acids would generate a culture environment which is so stressful that necrosis becomes the dominant form of cell death. Moreover, one would anticipate that the extent to which Bcl-2 would delay death in such an environment would be minimal. However, as shown in Table 2 the level of protection was indeed highly significant. The level of protection provided even in the complete absence of amino acids was very surprising and again emphasises the fact that Bcl-2 over-expression can permit the cells to enter a state of very low metabolic activity.

CONCLUSION

The results of the present study demonstrated that over-expression of Bcl-2 in NS0 cells has substantially prevent the apoptotic cell death in serum and amino acid deprivation conditions. The *bcl-2* transfected cell lines have exhibited a more efficient in nutrient metabolism and less serum dependent characteristic compared to its control counterpart. This characteristic is very important

for the development of serum and protein free culture.

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