THE EFFECT OF *bcl-2* OVER-EXPRESSION ON HYBRIDOMA CELL LINE CULTURED IN DIFFERENT SERUM FREE MEDIA.

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ABSTRACT

One of the criteria for large scale production of therapeutics using animal cell lines is the ability to grow in and can be maintained in inexpensive, serum free defined medium. Serum free medium however, compromised by the high levels of cell death seen in bioreactors, which is mainly *via* apoptosis. Previous studies in our laboratory have demonstrated that apoptosis in bioreactors can be prevented by *bcl-2* over-expression in these cell lines. The robustness of the *bcl-2* transfected hybridoma (TB/C3) cell line could allow them to be grown in simple medium formulation especially without serum supplementation which could facilitate the down stream processing, overall manufacturing process and product consistency. In the present study, the impact of the suppression of apoptosis on cell adaptation to various media formulation was carried out. This cell line was successfully grown in suspension batch culture with WMC5, HybSFM, DMEM/F12, WMC5/RPMI 1640 media and in serial dilution of HybSFM without prior adaptation.

Keywords: bcl-2, serum free medium, apoptosis, hybridoma cell line

INTRODUCTION

Apoptosis was found to be a major mechanism of cell death in bioreactors of hybridoma cell line (Singh *et. al.*, 1994; Simpson *et. al.*, 1997). Rapid progress has been made in the identification of the factors that trigger apoptosis in bioreactors and the development strategies, which prevent this form of death (Singh *et. al.*, 1996). Transfection with the *bcl-2* gene, an acronym for the B-lymphoma/Leukemia-2, was initially discovered in a study of follicular non-Hodgkin's lymphoma has proved to be an effective strategy for the suppression of apoptosis in the bioreactor environment.

In our laboratory, the *bcl-2* gene has been successfully transfected into the TB/C3 hybridoma cell line (Simpson *et. al.*, 1997). This transfected cell line, termed TB/C3.bcl-2 has been to exhibit improved robustness in various environmental culture conditions known to induce apoptosis (Simpson *et. al.*, 1998; Perani *et. al.*, 1998; Fassnacht *et. al.*, 1998; Bierau *et. al.*, 1998; Fassnacht *et. al.*, 1999; Simpson *et. al.*, 1999). Moreover, the suppression of apoptosis was also found to improve culture productivity (Fassnacht *et. al.*, 1998; Simpson *et. al.*, 1997).

In the present study, the impact of the *bcl-2* over expression in the suppression of apoptosis, on cell adaptation in various media formulation was carried out. This cell line was successfully grown in suspension batch culture with WMC5, HybSFM, DMEM/F12, WMC5/RPMI 1640 media and in serial dilution of HybSFM without prior adaptation. Furthermore, the glycosylation patterns of IgG1 from both *bcl-2* transfected and control TB/C3 cell lines in HybSFM (purified IgG1 by HPAEC-PAD) and in WMC5/RPMI 1640 (unpurified IgG1 by lectin blotting) were found to be similar.

MATERIALS AND METHODS

Chemicals and supplements

Animal cell culture grade, electrophoresis grade and HPLC grade chemicals used in this study were obtained from Sigma Chemicals, Poole, UK unless otherwise stated. All the solutions were prepared using de-ionised double distilled water (Millipore Corp., USA). The foetal calf serum (FCS) and Ultra-low IgG FBS (foetal bovine serum) used were supplied by Gibco-BRL, Paisley, U.K.

Cell culture media preparation

RPMI 1640, Hybridoma-SFM and DMEM were supplied by Gibco-BRL, Paisley, UK. DMEM/F12 was purchased from Sigma Chemicals, Poole, UK. All media were 0.2μm sterile filtered, stored at 4 °C and warmed to 37 °C prior to use.

WMC5 medium. This medium is a proprietary medium formulation, especially for the cultivation of CHO 320 cell lines and was a kind gift from the Wellcome Foundation, UK. The medium was prepared by dissolving 214g of WMC5 powder, 30.24g sodium bicarbonate, 50mg recombinant insulin (Recombulin was initially dissolved in 10ml 0.01M HCl) and 1.13mg methotrexate (MSX) (initially dissolved in a few drops of KOH) in 9L of deionised double distilled water. Once dissolved, water was added to take the volume up to 10L. Medium was filtered using a glass-fibre pre-filter and a Sartobran 300 filter unit (Sartorius).

Combination medium, RPMI 1640/WMC5. This combination medium was prepared by the addition of 1 part of basal RPMI 1640 to 1 part of WMC5 medium. To this mixture, 20μg/ml transferrin, 10μg/ml insulin, 360μg/ml glutamine and nucleotides (30μM each of adenosine, guanosine, cytidine, thymidine and uridine; all sterile filtered) were added.

Hybridoma-SFM and its dilutions. The medium was purchased pre-prepared in a 1L liquid form. The serial dilutions (1/2, ¼ and 1/10) of this medium were made by adding an appropriate volume of hybSFM to basal RPMI 1640

Cell lines and their maintenance

The mammalian cell lines used were TB/C3 hybridoma cells (was cloned from WC3 cell line and was a kind gift from Professor R. Jefferis, Immunology Dept., Birmingham University, UK). This cell line was transfected in previous work of Simpson *et. al.*, (1997) either with *bcl-2* carrier plasmid, pEF-bcl2MC1ncopA or control plasmid, pEF-MC1neopA (Visvader *et. al.*, 1992). The TB/C3 cell line is an NS1-derived hybridoma, which produces immunoglobulin G subclass 1 (IgG1) monoclonal antibody.

RESULTS

The growth characteristics, mechanism of death and productivity of hybridoma cell lines in hybSFM

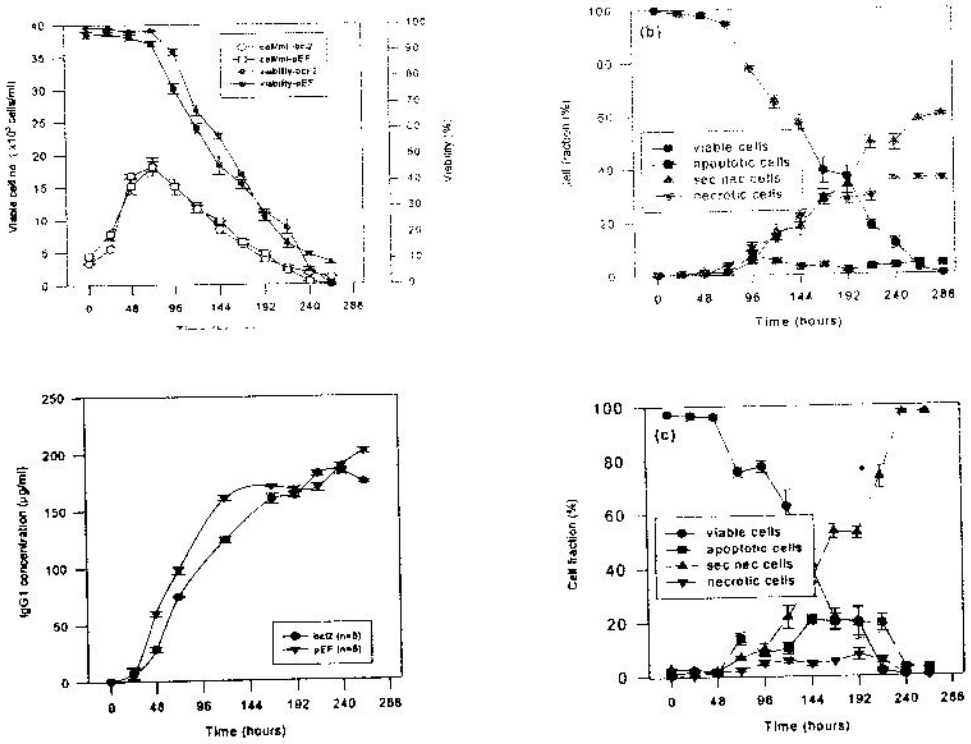

Fig 1: The growth characteristic of bcl-2 transfected and contol hybridoma cell lines in

The investigation was initiated by growing both *bcl-2* transfected and control hybridoma cells in hybridoma serum free medium. The viable cell density and viability of both cell cultures are presented in Figure 1a. The viable cell density of *bcl-2* transfected and control cultures and the viability of these two cultures were similar but the mechanism of death was somewhat different. In the control culture (Fig 1b), cell death was by apoptosis, but in the *bcl-2* cell culture (Fig 1c) was predominantly by necrosis. The productivity of both cell lines was also similar (Fig 1d).

To establish whether the medium used was responsible for the identical growth patterns of the two cell lines, a series of diluted medium were prepared and growth characteristics determined. Four sets of suspension batch cultures were run in parallel using non-diluted hybSFM, 50%, 25% and 10% hybSFM diluted in RPMI 1640. The cell density of both *bcl-2* transfected and control cell lines grown in each medium preparation is presented in Figure 2. There was no significant difference in viable cell density between both, *bcl-2* transfected and control hybridoma cell lines grown in non-diluted hybSFM (Figure 2a) and in 50% hybSFM (Figure 2b). In the 25% diluted hybSFM (Figure 2c), a significant difference in death rate was observed between the *bcl-2* transfected and the control cultures where, viable cell density declined at a comparatively lower rate in the *bcl-2* culture to control culture.

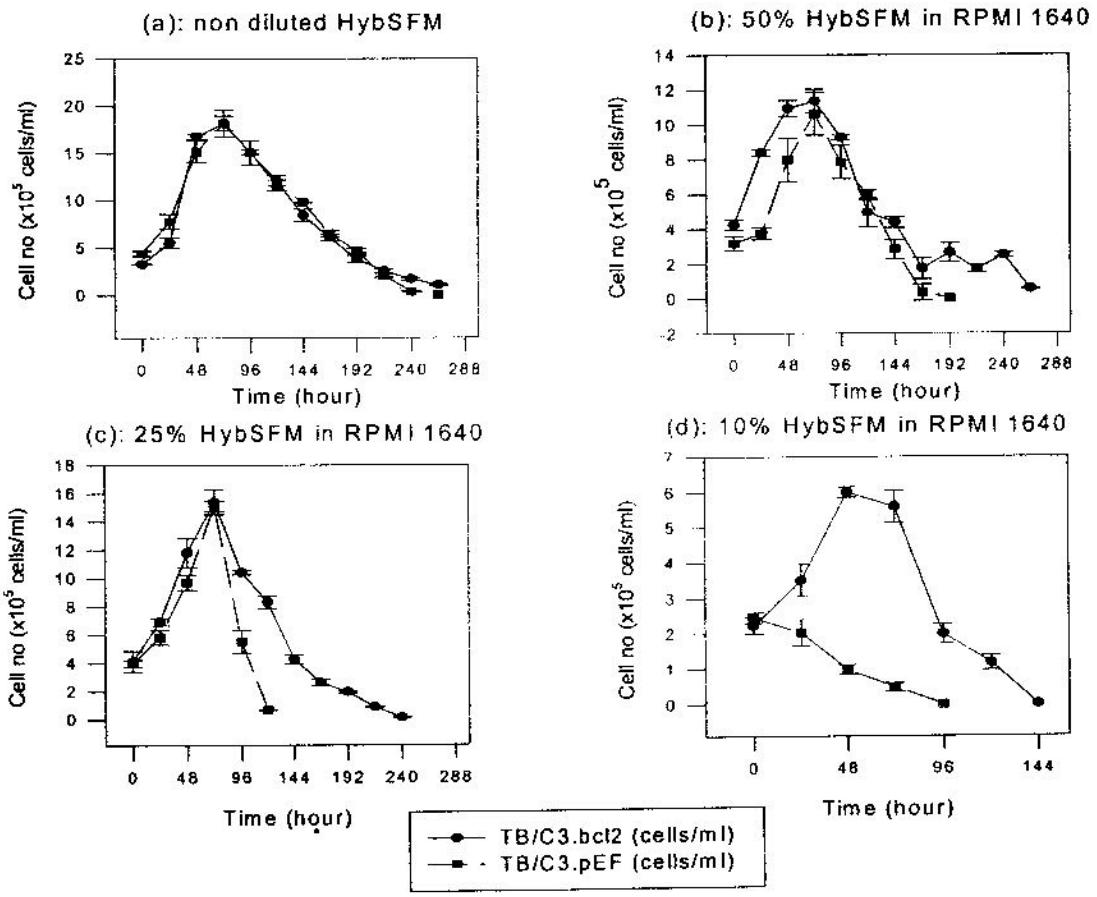

Fig 2: The growth characteristics of bcl-2 transfected and control hybridoma cell lines in diluted HybSFM.

In the extended experiment using 10% hybSFM (Figure 2d), the viable cell density of *bcl-2* transfected hybridoma increased to maximum 48 hours post inoculation. The viable cell number

gradually declined and reached zero after 144 hours post inoculation. In the control culture, the viable cell number declined immediately and reached zero after 96 hours post infection.

The growth characteristics of hybridoma cell lines grown in DMEM/HAMF12 medium.

The impact of *bcl-2* over-expression on hybridoma cell growth in the absence of serum was assessed using basal medium of DMEM/HAM-F12. As shown on Figure 3a, the control culture reached zero viability by day 3. By contrast, *bcl-2* transfected cells exhibited slow growth in sup-optimal environment and increased in viable cell after 5 days. The viability of the culture was increased to 90% within 2 days of inoculation and gradually declined on day 7.

In the second experiment, 1% serum concentration was added to the cultures (Figure 3b). The viable cell number of both cell lines increased to maximum. In the *bcl-2* transfected culture, the viable cell number was still above 3 x 10^5 cells/ml after 11 days (at which stage the experiment was terminated) but in control culture, it reached zero after 6 days. The viability of the control culture rapidly declined to zero on day 6 while in *bcl-2* transfected culture viability gradually declined to 40% on day 11.

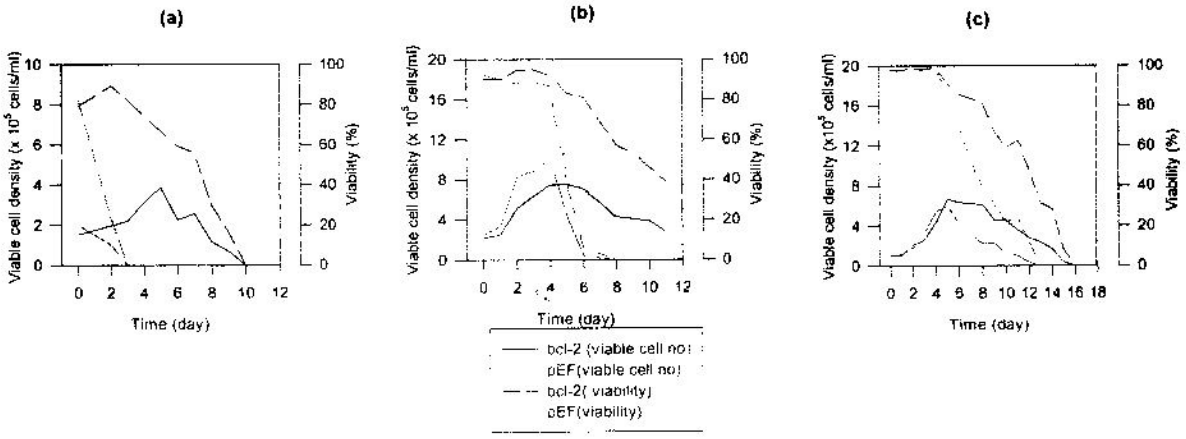

Fig 3: Growth characteristics of bcl-2 transfected and control hybrisoma cell lines in reduced serum supplemented DMEM/HAM F12

Finally, both *bcl-2* transfected and control hybridoma cell lines were grown in DMEM/HAM-F12 medium with 2.5% FCS suspension batch culture (Figure 3c). The viable cell density increased for control culture and *bcl-2* transfected culture after 5 days post inoculation. However, viable cell density of the control culture declined rapidly and reached zero on day 13. The viable cell density of *bcl-2* transfected culture remained above 6 x 10^5 cells/ml and finally declined to zero after 16 days post inoculation. The viability of these cell lines remained above 98% for 5 days from post inoculation point. The viability of the control culture declined drastically while *bcl-2* transfected culture decreased at much slower rate.

The growth characteristics of hybridoma cell lines grown in WMC5 medium and in WMC5/RPMI 1640 medium (with supplements).

The viable cell density and viability of both hybridoma cell lines grown in WMC5 medium is presented in Figure 4a. In this experiment, the viable cell density of *bcl-2* transfected hybridoma culture was increased and gradually decline after 48 hours post inoculation. In contrast, the viable cell number of the control culture declined immediately.

The viable cell density and viability of hybridoma cell lines grown in WMC5/RPMI 1640 medium (with supplements) is presented in Figure 4b. The viable cell density of both cell lines increased after 24 hours post inoculation. Interestingly, viable cell density of *bcl-2* transfected culture

reached maximum of 22 x 10^5 cells/ml after 72 hours. The viable cell density of control culture however remained constant at around 8 x 10^5 cells/ml until 96 hours. The viability of *bcl-2* cultures remained above 95% up to 72 hours and only declined slightly to 85% after 96 hours. In contrast, the viability the control culture declined gradually 24

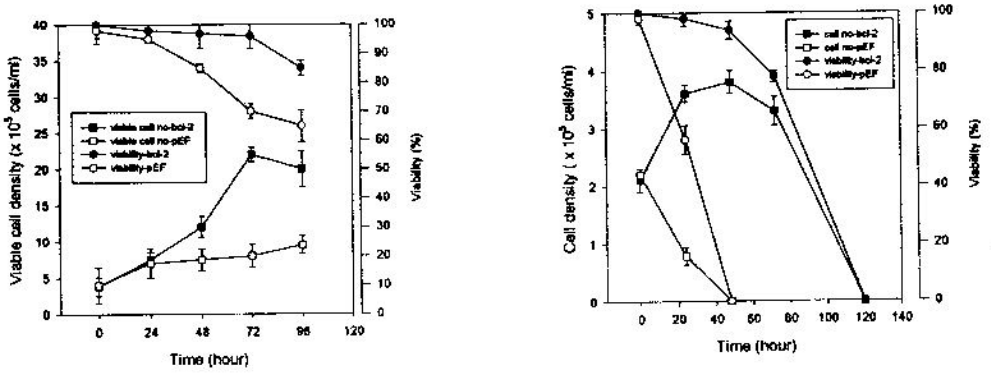

Fig 4: The growth characteristics of *bcl-2* transfected and control hybridoma cell lines grown in (a) WMC5 medium and in (b) WMC5/RPMI 1640 medium (with supplements).

DISCUSSIONS

The impact of bcl-2 over expression on hybridoma cell lines grown in HybSFM

An investigation on the impact of *bcl-2* over-expression in serum free medium was conducted in this work. The results in Figure 1 clearly indicate that in optimal growth condition (using a propriety serum free medium, hybSFM) *bcl-2* gene in transfected culture does not play an important role in extending cell viability. It was found that both cell lines have similar growth characteristics (Figure 1a) and productivity (Figure 1d) despite having a different mechanism of cell death (Figure 1b and 1c). The rich nutritional environment has probably extended the growth of both cultures to more than 240 hours. The mechanism of cell death in the two cell lines was found to be significantly different (Figure 1b and 1c). The *bcl-2* cell lines were found to experience death by necrosis and apoptosis while control cell lines underwent cell death solely by apoptosis. The death pattern is to confirm the over expression of *bcl-2* gene in the *bcl-2* transfected hybridoma culture.

In the second experiment, several tests on the growth characteristics of both cell lines in serially diluted hybSFM were carried out. The results in Figure 2 clearly confirmed that the medium was a rich formulation since both cell lines showed similar growth characteristics when grown in 50% hybSFM. The impact of *bcl-2* over-expression in extending cell viability in the TB/C3.bcl-2 cell lines was only significant when both cell lines were grown in 25% and 10% hybSFM suspension batch cultures.

The impact of bcl-2 over expression on hybridoma cell lines grown in DMEM/HAM-F12

Depending on the type of medium utilised to grow hybridoma cell lines, serum was usually added to the medium as a growth supplement in the concentration range, 5 to 10% (v/v). The ability to grow producer cell lines in negligible concentrations of serum is an important objective for the production of biopharmaceuticals. The robustness of the *bcl-2* transfected TB/C3 hyridoma cell line was also tested in DMEM/HAM-F12 medium. The results in Figure 2 clearly show that *bcl-2* cells were able to grow without adaptation in basal DMEM/HAM-F12 medium, whilst control cells failed to grow in this medium (Figure 2a). The results in these present study shows that *bcl-2* transfected cells could effectively maximised the limited nutrients and essential components in this basal DMEM/HAM-F12 medium for cell growth. These *bcl-2* transfected cells could have reduced the nutrients utilisation rates in this basal medium as previously suggested by Simpson *et. al.*, 1998.

Interestingly, when a small amount of serum (1% in Figure 2b and 2.5% in Figure 2c) added to the medium, both cell lines were able to grow and increase their maximum cell number by nearly twofold in comparison to the cells grown in basal medium. More importantly, the viability of the *bcl-2* cell lines in the serum supplemented DMEM/HAM-F12 was significantly extended.

The impact of bcl-2 over expression in WMC5 and in WMC5/RPMI 1640 combination media

The WMC5 medium was formulated for growth of CHO 320 cell lines producing IFN-γ. When *bcl-2* transfected TB/C3 hybridoma cell lines were tested for their robustness by cultivation in this medium without adaptation, the cell number and viability increased despite an immediate cell death of the control cell lines (Figure 3). WMC5 medium was formulated for growing CHO 320 cell lines. Similar to the growth characteristic of hybridoma in DMEM/HAM-F12, the supplements in WMC5 medium may be inadequate for growing hybridoma cell lines. The minimum supply of nutrients could have been the factor failing the control hybridoma cells to grow and proliferate. In contrast, *bcl-2* transfected cells could effectively maximised the limited nutrients supplied in the medium by reducing the nutrients utilisation rate as was previously found in the work of Simpson *et. al.*, 1998.

Another medium used to study the ability of *bcl-2* cell lines to grow in serum free medium was a combination medium of 50/50 WMC5 and RPMI 1640. The results in Figure 4b clearly show the ability of these cells to grow without adaptation in this medium.

CONCLUSIONS

The *bcl-2* transfected TB/C3 hybridoma cell lines are very robust. Over-expression of *bcl-2* aids growth of hybridoma cell lines in commercially available serum-free media tested. The ability of *bcl-2* transfected hybridoma cell line to grow in a new medium without adaptation will reduce production time. Able to utilised minimal supplements for growth in certain media will reduced production cost.

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