Effect of *Bcl-2* Expression on Hybridoma Cell Growth in Serum-Supplemented, Protein-Free and Diluted Media

D. Fassnacht¹, S. Rössing¹, F. Franěk², M. Al-Rubeai³ & R. Pörtner¹

¹ Technische Universität Hamburg-Harburg, Bioprozeß- und Bioverfahrenstechnik, Denickestraße 15, D-21071 Hamburg, Germany; ² Institute of Molecular Genetics, Czech Academy of Science, CZ-14220 Praha 4, Czech Republic; ³ Center for Biochemical Engineering, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham, B15 2TT, U.K.

Received 18 August, 1997; accepted 5 December, 1997

Key words: apoptosis, *Bcl-2*, diluted medium, hybridoma, protein-free medium

Abstract

Two transfected hybridoma cell lines TB/C3-bcl2 (overexpressing the *Bcl-2* protein) and TB/C3-pEF (control cell line), were compared in batch suspension cultures using a medium supplemented either with horse serum or with a protein-free, iron-rich supplement. The membrane intact index (percentage of cells with intact membranes determined by trypan blue staining) of the TB/C3-bcl2 cell line decreased much slower than that of the control cell line during the dying phase of the cultures. No significant difference in antibody, lactate and ammonia production as well as glucose and glutamine consumption was noted in the exponential phase of the experiments. Both cell lines were also compared in batch experiments using media diluted with saline to further investigate the effect of *Bcl-2* under sub-optimal conditions. The *Bcl-2* overexpressing cell line again exhibited a higher membrane intact index at increasing dilution steps.

Introduction

The production of antibodies by hybridoma cultures is well established in industrial processes, and considerable progress in the optimization of these in laboratory scale has been achieved in recent years. This is mainly due to new fermentation strategies such as fed-batch (Bushell et al., 1994; Pörtner et al., 1996; Zhou et al., 1995), dialysis (Pörtner et al., 1994; Amos et al., 1994), immobilization (Ong et al., 1994; Pörtner et al., 1997; Fassnacht et al., in press) or both immobilization and dialysis (Bohmann et al., 1992; Bohmann et al., 1995). Other approaches improve and extend cultivation by optimizing the medium contents. This is either achieved by a general addition of nutrients (Fassnacht et al., 1997; Franěk, 1995; Franěk et al., 1997; Franěk and Šrámková, 1996) or a specific supplementation with consumed nutrients only (Duval et al., 1991; Hiller et al., 1994), which requires detailed studies of the metabolism for each cell line. Another possibility to further increase productivity is recombinant DNA

technology which permits specific metabolic engineering such as the regulation of proliferation (Lee et al., 1997) or programmed cell death (apoptosis) (Simpson et al., 1997; Itoh et al., 1995; Singh et al., 1997).

The process of apoptosis has been extensively studied in recent years (Kerr and Harmon, 1991; Cotter and Al-Rubeai, 1995). In contrast to cell death resulting from physical injury (necrosis), which is typified by cytoplasmic organelle destruction and loss of membrane integrity, apoptosis is an active genetically controlled process of cell death. A wide variety of triggers can induce this, examples include deprivation of survival factors, presence of specific death factors, environmental stress such as DNA damage or glutamine and glucose limitation. A series of changes within the cell take place once apoptosis is initiated such as the condensation and fragmentation of the chromatin, the cytoplasmic boiling as well as an activation of endonucleases which cleave the DNA into approx. 180 base pair oligomers (Franěk and Dolníková, 1991; Franěk et al., 1992).

During biotechnological processes, such as the cultivation of hybridoma cells where cell death occurs mainly by apoptosis at the end of the growth phase in batch cultures, resulting in high death rates, it can limit culture productivity. The sub-optimal conditions which induce apoptosis like the deprivation of substrate also exist in continuous cultures where the highest productivity is usually obtained at low dilution rates. It is therefore of commercial interest to suppress apoptosis and prolong the production of antibodies.

One way to suppress apoptosis is the transfection of relevant cell lines with vectors producing various proteins which regulate apoptosis. *Bcl-2* (Vaux et al., 1988; Hockenberry et al., 1990), the thoroughly studied product of an oncogene, has been demonstrated to suppress apoptosis in various cell lines. The detailed mechanisms are not yet all clear, but *Bcl-2* seems to interact with other proteins of the *Bcl-2* family (Oltvai et al., 1993) such as *Bax* or *Bak*, suggesting that the relationship between the proteins is an antagonistic one.

The aim of this work was to investigate the effect of *Bcl-2* overexpression in a hybridoma cell line on metabolism and death under stressful conditions. The conditions evaluated were the death phase of batch suspension cultures using media supplemented either with serum or with a protein-free supplement and growth in diluted media. All experiments were carried out with a *Bcl-2* transfected cell line as well as a control cell line. In order to supplement the work of Simpson et al. (1997) the same transfected cell lines TB/C3-bcl2 and TB/C3-pEF were used for the experiments.

Materials and methods

Cell line and growth condition

Two transfected NS1-derived mouse hybridoma cell lines TB/C3-bcl2 and TB/C3-pEF, both producing IgG antibodies against an antigenic determinant in the hapten C γ 2 domain in the Fc region of human IgG, were used in this study. These cell lines were transfected with the *Bcl-2* (pEF bcl2-MCIneoA) and control (pEF-MCIneoA) plasmids by Simpson et al. (1997).

For growth, a 1:1 mixture of Iscove's MDM and Ham's F12 supplemented with 2 mmol l⁻¹ L-glutamine, 2 g l⁻¹ NaHCO₃, 0.01% PEG and 50 mmol l⁻¹ ethanolamine was used as basal medium. This basal medium was either supplemented with 1% (v/w)

of a protein-free iron-rich supplement (IR) containing 20 mmol l⁻¹ ferric citrate (Franěk and Dolníková, 1991) or 3% (v/w) horse serum (HS). The total amount of glutamine in both media was 3 mmol l⁻¹ and 15 mmol l⁻¹ for glucose.

Batch experiments

For the batch experiments, cultivation took place in 25 cm² stationary flasks at 37 °C in a 5% CO₂ humidified atmosphere. The flasks were inoculated with exponentially growing cells at an initial cell density of 1 · 10⁵ cells ml⁻¹. Growth curves were obtained by removing a doublet of flasks each day for counting and analyzes.

Diluted media

The effect of nutrient limitation was investigated in cultures conducted in IR media diluted to various degrees with saline. The medium was mixed at defined ratios with PBS (phosphate buffered saline) containing the IR supplement in the same proportion as the IR basal medium. The cultures were set up in 25 cm² stationary flasks as described before. Viable cell density and membrane intact index were determined after 72 hours. The relative viable cell density is defined as the percentage of the viable cell density at a particular medium dilution divided by the viable cell density in the undiluted medium. Three independent experiments were carried out.

Analytical methods

Glucose and lactate were determined with a YSI-Analyser 2700 (YSI, USA). A relative error of 2% can be assumed (Pörtner and Schäfer, 1996; Hiller et al., 1993). Glutamine and ammonia were analyzed enzymatically (5% relative error), monoclonal antibodies by an IgG ELISA (all kits from Boehringer Mannheim, Germany) with a relative error of approx. 10%.

The total cell number and membrane intact index (percentage of membrane intact cells) were evaluated using haemocytometer counts with trypan blue exclusion. A relative error of 7% can be assumed (Pörtner and Schäfer, 1996).

DNA gel electrophoresis and sample preparation

After the centrifugation of the samples (3.5 · 10⁵ cells) the cell pellets were resuspended in 100 μ l PBS and

mixed with 100 μg RNase A (Boehringer Mannheim, Germany) purified of DNase by heating at 70 °C for 10 min. Phenolization was started after one hour of incubation at room temperature. The first extraction was performed with 100 μl sample, 50 μl phenol liquefied and washed in Tris buffer (Biomol, Germany) and 50 μl chloroform/iso-amyl alcohol (24:1). The second step was an extraction with the aqueous supernatant of the first extraction and 100 μl chloroform/iso-amyl alcohol (24:1). The DNA in the obtained aqueous supernatant was concentrated by precipitation with 10 volumes of isopropanol and 1 volume of sodium acetate (3 mol l^{-1} , pH 5.2) for 10 minutes at -20°C followed by two rinsing steps with 70% ethanol. After drying, the DNA pellets were resuspended in 20 μl sterile Tris-EDTA-buffer.

For electrophoresis a 1% agarose-gel containing 0.005% ethidiumbromide was prepared in Tris-acetate-EDTA-buffer. 5 μl of the samples was mixed with 0.5 μl gel-loading-buffer (40% saccharose, 0.25% bromphenol blue, 20 mM EDTA) and loaded directly onto the gel. 1.2 μl of the marker VII (Boehringer Mannheim, Germany) was used as a standard. The separation took 90 minutes at a voltage of 100 V. The DNA bands were visualized and photographed with UV-light.

Results

Adaptation to serum-free medium

The first task was to adapt both cell lines to protein-free medium. This was performed by adding 1% of the iron-rich supplement (IR) to the medium with 3% horse serum (HS) and then slowly reducing the level of HS when sub-culturing the cell lines. This was well accomplished for the TB/C3-bcl2 cell line which grew well in the IR medium after 3 weeks. On the other hand it took over 2 month to adapt the control cell line TB/C3-pEF.

Comparing the cell lines in batch cultures

The main objective of comparing the two cell lines in batch cultures was to verify the effect of *Bcl-2* during ideal growth conditions (exponential phase) as well as in sub-optimal conditions (death phase). These experiments were performed with two different medium supplements: 3% HS and 1% IR to clarify if the medium supplement has an effect on the behavior of the cell lines.

Figure 1. Total (up triangles) and membrane intact cell densities (down triangles) of batch cultures of TB/C3-bcl2 (closed triangles) and TB/C3-pEF (open triangles) using two different medium supplements, 3% HS and a protein-free iron-rich supplement IR, respectively.

The two cell lines grew in both media to the same maximal cell density of approx. $1.6 \cdot 10^6$ cells ml^{-1} (Fig. 1). This density was reached after 72 hours when using the HS medium and after 96 hours for the IR medium. The difference is due to a longer lag-phase in the IR medium. After the maximum cell density was reached, the cells started to die and loose their membrane integrity, while the total cell density remained constant or decreased only slowly during the death phase. In the HS medium, *Bcl-2* seemed to prolong the exponential phase and to delay the cell death for 48 hours compared to the TB/C3-pEF cell line. However, the viable cell number declined with the same speed, once cell death became prominent. The growth of both cell lines in the IR medium did not only show the delay of the death phase but also a significant reduction in the specific death rate for the TB/C3-bcl2 cell line. Fifty percent of the TB/C3-bcl2 cells in the IR medium

Figure 2. Glucose (up triangles) and lactate (down triangles) concentrations of batch cultures of TB/C3-bcl2 (close triangles) and TB/C3-pEF (open triangles) in the two media.

still had intact membranes after 9 days of cultivation, whereas after this time no viable cells were found for all three other experiments described here.

There was no significant difference in glucose and glutamine consumption as well as lactate and ammonia production for the exponential phases of the experiments (Fig. 2 and Fig. 3). In all batch experiments ammonia reached a concentration of 3-4 mmol l⁻¹ which can not be regarded as inhibiting (Lüdemann et al., 1994). The limiting substance is yet to be identified. The glutamine concentration was approx. 0.5 mmol l⁻¹ at the end of the exponential phase for all experiments. The same was true for glucose which only decreased to 3 mmol l⁻¹ for all but one experiment. Both concentrations can not be considered as limiting. Two fed-batch experiments, feeding only a concentrated glucose and glutamine solution were per-

Figure 3. Glutamine (up triangles) and ammonia (down triangles) concentrations of the first days of cultivating TB/C3-bcl2 (close triangles) and TB/C3-pEF (open triangles) in the two media.

Figure 4. Antibody concentrations of the batch cultures of TB/C3-bcl2 (up triangles) and TB/C3-pEF (down triangles) in IR medium (open triangles) and HS medium (closed triangles).

Figure 5. DNA gel electrophoresis at different days of four different batch culture experiments to determine the character of cell death. Both cell lines and media were investigated. The marker is at the center of the gels.

formed to verify that the growth arrest is not due to glutamine or glucose limitation (data not shown). Both cell lines were grown in the IR medium in 400 ml suspension reactors. Feeding was initiated when glutamine reached a concentration of 1 mmol l^{-1} . The glutamine and glucose concentrations started to increase during the next 12 hours of feeding, but the cell growth arrested at the same cell-density than in the batch experiments. Therefore, some other substrate must be the cause for the inhibition of growth in the used media, such as an amino acids or vitamin.

An insignificantly higher total antibody concentration (Fig. 4) of 55 mg l^{-1} is notable for the TB/C3-bcl2 cell line in both media compared to the control cell line where 45 to 50 mg l^{-1} were obtained. The antibody concentration of both cell lines increased with the same speed during exponential growth for the IR medium. In the HS medium the increase of the antibody concentration of the *Bcl-2* overexpressing cells was slower than for the control cell line, but the TB/C3-bcl2 could catch up in the death phase due to higher membrane intact cells.

DNA gel electrophoresis was performed for the experiments (Fig. 5) to examine the effect of *Bcl-2* on the mechanism of cell death (i.e. whether it is necrotic or apoptotic). The DNA of cells, undergoing apoptosis, is cleaved by endonucleases into oligomers of approx. 180 base pairs. These oligomers reveal a typical DNA ladder on the electrophoresis gel which was visible in all of the experiments. Therefore *Bcl-2* does not prevent apoptosis totally, but rather slows this process down.

Comparing the cell lines in diluted media

These experiments were carried out in diluted media in order to compare the two cell lines under nutrient limitation. Both hybridoma cell lines reached a viable cell density of $6 \cdot 10^5$ to $7 \cdot 10^5 \text{ cells ml}^{-1}$ in the undiluted IR medium after 72 hours. The membrane intact index was close to 90%. The reduction in the content of nutrients, achieved by medium dilution, resulted both in a lower relative viable cell density and in a lower membrane intact index (Fig. 6). The TB/C3-bcl2 cell line displayed higher resistance to the starvation stress.

Figure 6. Relative viable cell density and membrane intact index of the cell lines TB/C3-bcl2 (closed circles) and TB/C3-pEF (open circles) in IR media diluted with saline.

At the verge of starvation-induced death, i.e. in 40% to 50% medium, the values of the membrane intact index clearly documented the superiority of the TB/C3-bcl2 cell line.

Discussion

The results obtained from the batch experiments show that both transfected cell lines have a comparable metabolism during exponential growth. This indicates that no selection of the two cell lines took place after transfection and therefore differences between the cell lines are only due to the overexpression of *Bcl-2*. The total antibody production of the TB/C3-bcl2 cell line in the batch experiments was slightly higher than that of the control cell line. This is mainly due to the slower decrease in the membrane integrity of the TB/C3-bcl2 cells, not to a higher antibody production rate during exponential growth. The greatest effect of *Bcl-2* was the increase in culture longevity, especially when us-

ing the protein-free IR medium where 50% of the cells still showed intact membranes after 9 days of cultivation. The overexpression of *Bcl-2* however, showed no increase in proliferation. In accordance with Itoh et al. (1995), it was found that *Bcl-2* does not keep the cells from undergoing apoptosis, but that this process is slowed down. It is also notable that the TB/C3-bcl2 cell line showed a different behavior in the two media during the death phase. An influence of medium components on the death phase was already observed for other hybridoma cell lines (Fassnacht et al., 1997; Franěk, 1995).

The advantage of the TB/C3-bcl2 cell line can also be seen when comparing the cell lines under nutrient limitation in diluted media. *Bcl-2* slows the apoptotic process down resulting in an higher membrane intact index. During the adaptation to the IR medium, a stable cultivation with the *Bcl-2* overexpressing cell line was obtained significantly faster than with the control cell line.

Conclusions

The overexpression of *Bcl-2* did not have an influence on the metabolism of cells growing under ideal conditions (exponential phase of batch cultures). The *Bcl-2* overexpressing cell line survived much better than the control cell line when induced to stressful conditions such as the stationary phase in batch experiments, growth in saline diluted media or the adaptation to protein-free media.

Acknowledgments

This project is partly financed by the Volkswagen Stiftung (grant no.: I/71230) and the EC project 'Increasing cell culture productivity by control of programmed cell death (apoptosis)' (grant no.: 950207).

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Address for correspondence: R. Pörtner, Technische Universität Hamburg-Harburg, Bioprozeß- und Bioverfahrenstechnik, Denickestraße 15, D-21071 Hamburg, Germany. E-mail: Poertner@tu-hamburg.d400.de