BHRF-1 as a Tool for Genetic Inhibition of Apoptotis in Hybridoma Cell Cultures

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Abstract: Cell death is a critical factor in hybridoma cell cultures. At high cell densities, the limitation of nutrients, mitogens and oxygen leads to elevated death rates and to the accumulation of cell debris affecting the quality of the product and downstream processing, therefore compromising the productivity and cost-effectiveness of the bioprocess. In the present work, we engineered a hybridoma cell line to express either the antiapoptosis gene Bcl-2 or its viral homologue BHRF-1 (isolated from Epstein Barr virus) and analyse their effects on the improvement of cell viability and productivity under different cell culture strategies as batch, continuous and perfusion.

Key words: Hybridoma, Apoptosis protection, Metabolic engineering, BHRF-1, Bcl-2

1. INTRODUCTION

Our previous studies on the behaviour of the hybridoma KB26.5 under conditions of glutamine depletion evidenced the success of caspase-inhibitor treatment in the delay of the apoptosis programme and the capacity to recover those cultures when they were brought to normal growing conditions, i.e. complete medium (Tintó *et al*., 2002). However, the high cost of these molecules bars them from their use in large-scale cultures. For this reason, genetic strategies directed to control regulatory points upstream of effector caspases are desirable for batch, continuous and perfusion processes at industrial scale. Consequently, a particular attention has been given to the need to interfere in the apoptotic pathways at the level of the mitochondria, which play a central role in apoptosis integrating death signals through Bcl-2 family members and co-ordinating caspase activation through the release of apoptogenic factors that are normally sequestered in the mitochondrial intermembrane space (Figure 1). The involvement of several caspases in channelling

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the apoptotic signal indicates that potential genetic modifications should either consider more than one single target or be directed upstream to their activation, which is at the mitochondrial level.

2. MATERIALS AND METHODS

2.1 Cell line, medium and culture conditions

The KB26.5 murine hybridoma was cultured as described (Sanfeliu *et al*., 1997).

2.2 Plasmid constructs

Sequences encoding cDNA from Bcl-2 and BHRF-1 were isolated as described (Vives *et al*., 2002) and cloned into pcDNA3.1 (Invitrogen) and pIRES-neo/pIRES-puro2 (Clontech).

2.3 DNA delivery into hybridoma cells

Three methods were used: (1) lipofection with DMRIE-C reagent (Life Technologies); (2) electroporation as described in the protocol provided by BTX; and (3) calcium phosphate-mediated transfection by inclusion of adenovirus in coprecipitates (Lee and Welsh, 1999). Adenoviral particles (Ad2-CMV-GFP) were supplied by CBATEG (UAB, Bellaterra, Spain) at titers of approximately 5,2 x 10^{10} IU (Infectious Unit)/ml. In the optimisation process, we used pEYFP-1 (Clontech) to assess the efficiencies.

2.4 Hemagglutination assay

The presence of antibodies was determined by hemagglutination test (Sanfeliu, 1995).

2.5 Apoptosis detection

Cell viability, Annexin-V-Fluos positive cells and DNA fragmentation were measured as described (Tintó *et al*., 2002).

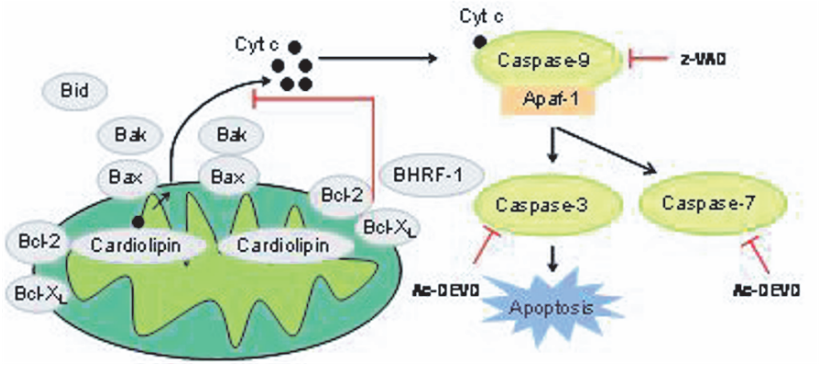

Figure 1. Model for the control of apoptosis and location of the Bcl-2 family members and caspase-inhibitors (z-VAD-fmk and Ac-DEVD-cho). Cytochrome c release is inhibited by anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-XL, and BHRF-1, while it is directly enhanced by the pro-apoptotic members such as Bak, Bax, and Bid. Once released from the mitochondria, Cytochrome c activates pro-caspase-9 via the apoptosome.

3. RESULTS AND DISCUSSION

3.1 Genetically engineered hybridoma cells

For most of animal cell lines, the two principal difficulties in the genetic engineering endeavour are their modest transfection efficiencies and the decline in the expression levels after several passages. We evaluated alternative approaches, such as the improvement of gene delivery methods and the use of plasmids based on bicistronic technology. The inclusion of adenovirus in DNA:CaPi coprecipitates (8%) provides by far the best results in comparison with eletroporation (1%) and lipofection (3%). Moreover, the maintenance of initial expression levels, and therefore antiapoptosis protection, was achieved with bicistronic vectors. In fact, our results showed at least 3 months stability of the transfectants, even in absence of selectable marker, allowing them to be employed in perfusion systems for long-lasting operational times.

3.2 Apoptosis protection in batch cultures

As reported previously, the use of protective members of the Bcl-2 family from viral origin, such as BHRF-1, not containing the Asp^{34} cleavage site of caspase-3, confer enhanced protection against apoptosis when compared to *bcl-2*- (Figures 2a and 2c) and *bclxl*-expressing cells (Vives *et al.*, 2003). Moreover, the use of bicistronic vectors harbouring *bhrf-1* substantially improved such protection in glutamine-deficient medium

(Figure 2d in comparison to 2b). *bhrf-1*-expressing cells not only exhibited the highest protection under glutamine-deficient culture conditions but also a decrease in viable cell number of only 15% at 72 hours, while the number of viable cells in the rest of the cultures at the same time showed a reduction in viability by more than 60%. Additionally, it was possible to recover the cultures even after being exposed to apoptosis-inducing conditions during significant time window, up to 72 hours, as demonstrated in continuous cultures (Figure 3).

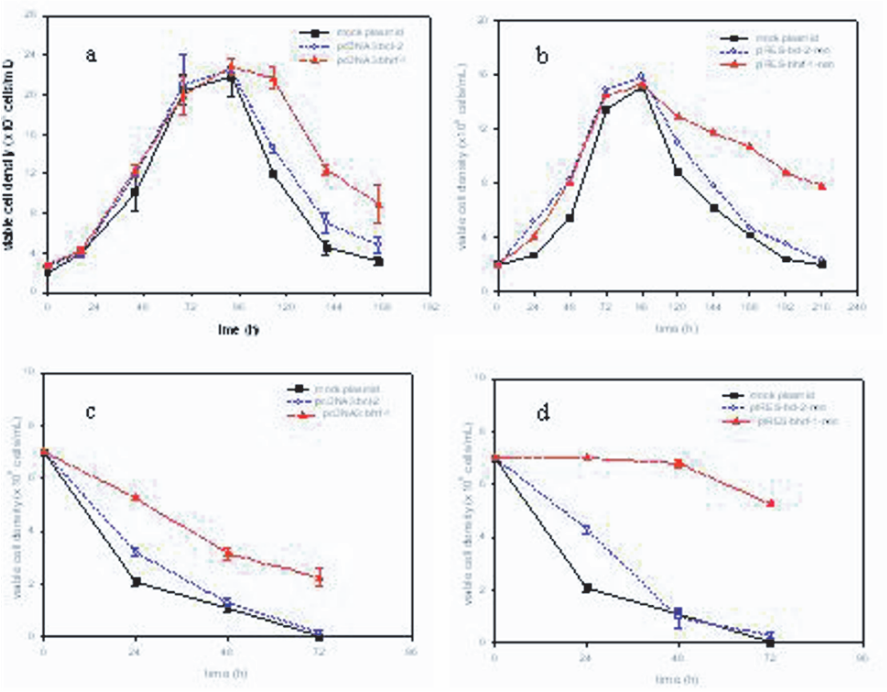

Figure 2. Effect of the expression of the antiapoptotic genes bcl-2 and bhrf-1 in batch cultures of hybridoma KB26.5. Cell growth profile of cells engineered using (a) monocistronic and (b) bicistronic vectors. Protection against apoptosis induced by glutamine deprivation in cultures of cells engineered with (c) monocistronic and (d) bicistronic vectors.

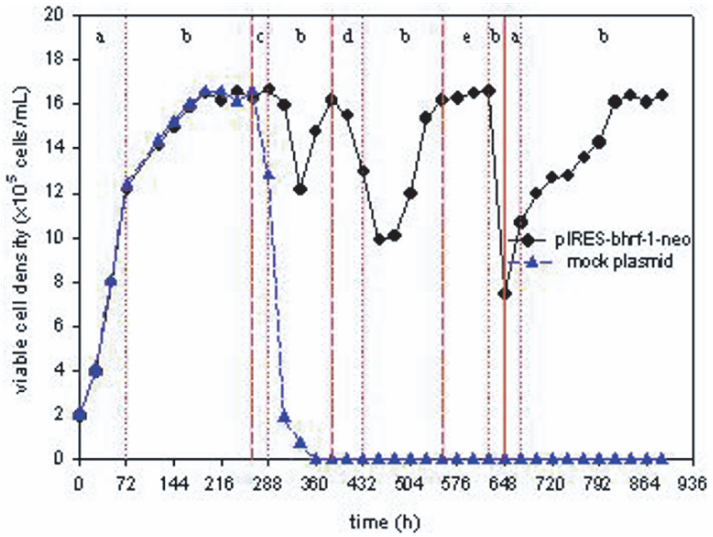

Figure 3. Continuous cultures of mock plasmid transfected (control) and bhrf-1-expressing hybridoma cells to evaluate the effect of the limitation of nutrients and the capacity to rescue cell viability. When the continuous culture was operated at a dilution rate of 0.16 h-1, the growth of both cell cultures was similar and reached the highest density at 264 h (a). At that point, continuous was suspended for 24 h and apoptosis was irreversibly triggered in the control culture coinciding with a fall in culture viability. By contrast, the specific growth rate of the bhrf-1-transfected cells could be quickly recovered when the system was operated again with elevated levels of cell survival after a momentary decline phase. As soon as cell densities were restored, another perturbation was applied to the system, for 48 h (d). This time, cell viability fell to 9.9 x 105 cells/mL, and they required a longer time to be recovered. Finally, a third perturbation was applied, for 72 h (e), and once more cells were recovered showing the protective effect conferred by the expression of bhrf-1.

3.3 Perfusion

High-density hybridoma perfusion cultures are extensively used for producing large amounts of MAbs. Although perfusion culture technology allows the maintenance of high cell concentration for extensive operational times, cell death is an important limitation: whereas viable cell density remains steady, accumulation of dead cells affects the quality of the product, downstream processing and clogging the perfusion system (Figure 4a). We performed perfusion cultures using mock plasmid transfected (control) and *bhrf-1*-expressing hybridoma cells and compared viable and dead cell numbers. Results show a dramatic reduction of dead cell number in *bhrf-1*-transfected cell cultures, 12.1 x 10^5 cells/mL vs 55.4 x 10^5 cells/mL (Figure 4b) in the control culture. Both batch and perfusion culture strategies are the preferred choice for the production of MAbs. However, they are

totally different systems. In batch cultures, the limitation of nutrients or oxygen triggers apoptosis in fairly extreme conditions. On the other hand, in perfusion cultures, although there could exist a limitation at elevated cell densities, triggering cell death and preventing to reach even higher concentrations, there are still resources available for every cell in the bioreactor. Under these conditions, mock transfected cultures sustain high percentage of cells in phase S, 50.1% at 192 h vs 47% at 96 h (in the middle of the exponential growth phase, Figure 4a). In contrast, cultures of *bhrf-1*-expressing cells show 36.3% at 192 h vs 53.9% at 96 h. Regarding the antibody production, *bcl-2*- and *bhrf-1*-expressing cells generate 85.2% and 87.5% with respect of mock transfected control, respectively. This slight reduction (less than 15%) in productivity is compensated by the fact that *bhrf-1*-expressing cells can be effective in long operational cultures and therefore it doesn't represent a drawback in their use in perfusion systems.

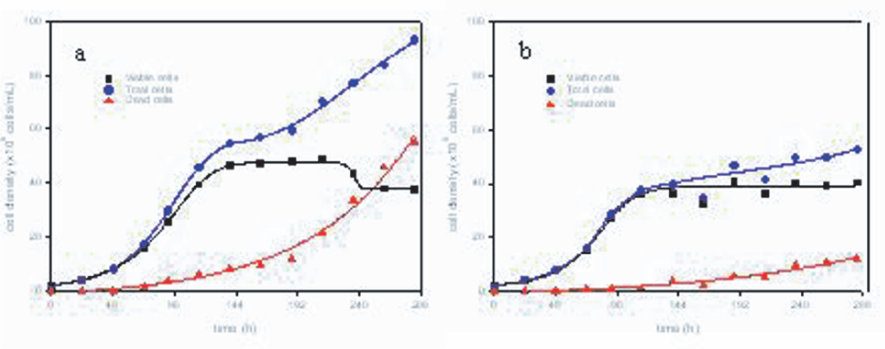

Figure 4. Perfusion cultures of mock plasmid transfected (control) (a) and bhrf-1-expressing hybridoma (b).

4. CONCLUSIONS

The performance and robustness of *bhrf-1*-expressing cells were tested under glutamine limitation in order to induce apoptosis as a consequence of starvation of this amino acid in batch cultures, interruption of continuous feeding in continuous cultures and depletion in perfusion operations. Under these conditions *bhrf-1*-expressing cells showed a high protection in front of the apoptotic process when compared to controls. It has been observed an increase in life span of 72 hours in batch and continuous experiments, and a reduction of almost 80% in cell death in perfusion operations. In addition, the protection conferred by BHRF-1 allowed the recovery of the cultures even after prolonged periods of time under apoptosis-inducing conditions,

therefore evidencing the potentiality of this technology for long operation periods in animal cell bioprocesses.

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