Characterization and fed-batch culture of hybridoma overexpressing apoptosis suppressing gene *bcl-2*

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Received 22 February 1996; accepted 25 September 1996

Key words: antibody productivity, apoptosis, *bcl-2*, fed batch culture, hybridoma

Abstract

Mouse hybridoma 2E3 transfected with human *bcl-2* gene survived longer with increasing expression level of *bcl-2* when cultured in DME medium supplemented with 9% serum. One of the transfectants, 2E3BCMGBcl-2, overexpressed *bcl-2* and could maintain viable cell density higher than the initial density for more than four days at a low 0.5% serum concentration. In comparison a mock transfectant 2E3BCMG remained viable for only one day. However, both hybridomas died out within a day in serum-free medium. These results suggested that *bcl-2* needed a small amount of some serum components to suppress apoptosis of the hybridoma. Overexpression of *bcl-2* also suppressed apoptosis of the hybridoma induced by glutamine deprivation. When hybridoma 2E3BCMGBcl-2 was inoculated in DME medium supplemented with 9% serum and cultured for 10 d with additional 2% serum feed at day 4 of the culture, viable cell density increased 2-fold and antibody produced 3-fold, in comparison with mock transfected 2E3 cultured in the same manner. The mock transfectant with additional feed of serum at day 4 of the culture showed no difference in viable cell density and antibody production. These results suggested that the mock transfectant committed to apoptosis before day 4 of the culture and the additional serum at day 4 could not reverse the commitment.

Introduction

The increasing demand for large quantities of monoclonal antibodies (MAbs), which have been widely used as reagents for clinical diagnosis and for assay of various compounds, requires the improvement of MAb productivity of *in vitro* hybridoma culture. MAb production of a culture increases when the viable culture period, that is, the growth and viable non-growth culture period is extended (Suzuki and Ollis, 1990; Takahashi *et al.*, 1994). However, hybridoma cells tend to die quickly after reaching the maximum cell density and hence the viable non-growth culture period is short as 2–3 d (Duval *et al.*, 1990; Perreault *et al.*, 1994; Vomastek and Franěk, 1993). Therefore, preventing cells from death which starts in the late exponential growth phase (Vomastek and Franěk, 1993) and

maintaining a viable batch culture for longer should increase MAb production of the culture. Hybridoma cells die due to depletion of nutrients such as amino acids (Franěk and Chládková-Šrámková, 1995) and glucose (Marcille and Massie, 1994; Singh *et al.*, 1994), limitation of growth factors such as serum components (Singh *et al.*, 1994) or some other conditions unfavorable for cells (Glacken *et al.*, 1988; Tohyama *et al.*, 1990). At least one of these cell-death inducing conditions occurs in the late exponential growth and stationary phases of batch culture. In general, cell death may follow two distinct patterns: necrosis and apoptosis, a mechanism of programmed cell death. Reportedly, the cell death of hybridomas in the late exponential growth and stationary phases of batch culture is mostly apoptosis (Marcille and Massie, 1994).

Figure 1a–b. Expression level of human bcl-2 in bcl-2 transfected hybridomas. The lysates from cells were boiled in SDS sample buffer and run on a 13% SDS-polyacrylamide gel. The proteins were electrotransferred to a nitrocellulose filter, then incubated with anti-bcl-2 antibody followed by treatment with peroxidase-conjugated goat anti-mouse Ig antibody. A) Lane 1 for mock transfectant 2E3MAM cultured without dexamethasone, Lane 2 and 3 for 2E3MAMBcl-2 cultured with or without 2 μ M dexamethasone, respectively. B) Lane 1 for mock transfectant 2E3BCMG, Lane 2 for 2E3BCMGbcl-2.

A method for preventing such cell death in batch culture is fed batch operation in which limiting components such as serum and glutamine are added to the culture in the late exponential growth phase. Another method is to modify cells to resist apoptosis. In our previous work (Itoh *et al.*, 1995), we transfected hybridoma 2E3 with human bcl-2 gene which is known to suppress multiple forms of apoptosis (Nunez *et al.*, 1990; Pattersson *et al.*, 1992; Tsujimoto 1989; Vaux *et al.*, 1988). Hybridoma 2E3BCMGbcl-2, the one transfected with bcl-2 using its BCMG vector, survived longer in batch culture than the source and mock transfected 2E3; the delayed emergence of the ladder pattern on electrophoresis of DNA extracted from

Figure 1b.

2E3BCMGbcl-2 cells indicated suppression of apoptosis (Itoh *et al.*, 1995).

In the present study, we combined these two methods, fed-batch culture and modification of cells to make them resistant to apoptosis, intending to prolong viable culture period for increasing MAb production per culture. We cultured hybridoma 2E3BCMGbcl-2 by fed batch operation, and obtained the intended results: prolonged viable culture period, two-fold increase in viable cell density, and three-fold increase of MAb production in comparison with the fed-batch cultured mock transfectant. We also examined the effect of bcl-2 expression level on survival of the cells, and the sensitivity of apoptosis of both 2E3 and 2E3BCMGbcl-2 to serum or glutamine limitation.

Materials and methods

Cell line and culture conditions

A cell line 2E3 employed throughout this study is a mouse hybridoma derived from a mouse myeloma P3X63 AG8U.1. The hybridoma 2E3 cells produce an

Figure 2. Effect of bcl-2 expression level on survival of cells. Hybridomas 2E3BCMGbc1-2, 2E3MAMbc1-2, and the corresponding mock transfectants were cultured in DME medium supplemented with 9% serum. (+) with 2 μ M dexamethasone.

IgG₁ specific to a trinitrophenyl (TNP)-hapten (Makishima *et al.*, 1992) at the rate of about 10 μ g/10⁶ cells day when growing at the specific growth rate of 1.0 to 1.4 day⁻¹.

Two bcl-2 expressing hybridomas, 2E3BCMGbc1-2 and 2E3MAMbc1-2, were prepared by transfecting hybridoma 2E3 with human bcl-2 gene inserted in vector BCMGSneo or pMAMneo, respectively, as described in our previous work (Itoh *et al.*, 1995). The corresponding mock transfectants, 2E3BCMG and 2E3MAM, were prepared similarly but without bcl-2 insertion.

These cells were cultured in DME medium (Nissui, Tokyo), supplemented with 9% FBS (vol/vol), 20 mM HEPES, 0.2% NaHCO₃, 2 mM glutamine, and 0.06 mg ml⁻¹ kanamycin, unless otherwise specified. The cells were grown in 25-cm² T-flask (Sumitomo Bakelite, Tokyo) at 37 °C in a humidified air containing CO₂ at 5%.

Viable and dead cells were determined by counting in a hemocytometer under a phase contrast microscope using trypan blue exclusion.

Western blot analysis of bcl-2 protein

Single-cell suspensions were lysed in 1% TritonX-100, 0.15 mM NaCl and 10 mM Tris (pH 7.4) with 50 μ g ml⁻¹ PMSF and 2 μ g ml⁻¹ aprotinin at 4 °C for 30 min. The cell lysates were boiled in SDS sample buffer for 5 min before being run on a SDS-polyacrylamide gel (13%). The lysate from 1 $\times$ 10⁵ cells was loaded into each lane. Gels were transferred to nitrocellulose filter overnight. Blots were blocked with 5% skim-milk for 2 h at room temperature. Human bcl-2 protein was detected by mouse anti-human-bcl-2 oncoprotein monoclonal IgG₁ (Boehringer Mannheim) to which peroxidase-conjugated goat anti-mouse Ig polyclonal antibody (Bio Source International, Inc.-Tago Products) was bound.

Determination of antibody concentration

The antibody concentration was determined by ELISA. MAb produced by cells was sandwiched by rabbit anti-mouse IgG polyclonal antibody (ZYMED Laboratories) and peroxidase-conjugated goat anti-mouse Ig polyclonal antibody (Bio Source International, Inc.-Tago Products). The amount of MAb

Figure 3. Growth and survival of bcl-2 overexpressing hybridoma cultured in serum limited medium. Both hybridoma 2E3BCMgbcl-2 and mock transfectant 2E3BCMg were cultured with or without serum. 2E3BCMg: (●) no serum, (■) 0.5% serum; 2E3BCMgbcl-2: (○) no serum, (□) 0.5% serum.

was determined by measuring absorbance, using o-phenylenediamine dihydrochloride as substrate for peroxidase. The known amount of the purified MAb secreted by 2E3 cells was used as standard.

Results/discussion

Effect of bcl-2 expression level on survival

The human bcl-2 protein in 2E3BCMgbcl-2 and 2E3MAMbcl-2 cells cultured with or without 2 μ M dexamethasone was assayed by western blotting (Fig. 1). The relative strength of the bcl-2 expression can be determined by comparing density of the bcl-2 band with that of the constitutively produced IgG bands in the same lane. In vector pMAMneo, the bcl-2 gene was inserted downstream MMTV-LTR promoter which was inducible by dexamethasone. Hence, the expression level of the bcl-2 in 2E3MAMbcl-2 cultured with dexamethasone was higher than that in the cell cul-

tured without the inducer, as indicated by comparison of Lane 2 with Lane 3 in Fig. 1-A, although the expression level was much lower than that in 2E3BCMgbcl-2 shown in Fig. 1-B.

To examine the effect of the expression level of bcl-2 on survival of the cells, we cultured the hybridomas 2E3BCMgbcl-2 and 2E3MAMbcl-2 as well as both mock transfectants with or without 2 μ M dexamethasone for those containing MMTV promoter. The viability data of these cultures are shown in Fig. 2. All the data of Figs. 2 to 6 were the results of each single run for each condition. Hybridoma 2E3BCMgbcl-2 but not the others survived distinctively longer. Combined with the result of the western blotting, this result indicated that bcl-2 needed its expression at a high level to improve survival of the cells. In the rest of this paper, the bcl-2 overexpressing cell represents 2E3BCMgbcl-2, and the control the mock transfectant 2E3BCMg. Among both mock transfectants 2E3BCMg and 2E3MAM and the source hybridoma

Figure 4. Growth and survival of bcl-2 overexpressing hybridoma cultured in glutamine limited medium. Both hybridoma 2E3BCMGbcl-2 and mock transfectant 2E3BCMG were cultured with or without 2 mM glutamine. 2E3BCMG: (●) no glutamine, (■) 2 mM glutamine; 2E3BCMGbcl-2: (○) no glutamine, (△) 0.2 mM glutamine, (□) 2 mM glutamine.

Figure 5. Growth and survival of bcl-2 overexpressing hybridoma in fed-batch culture. Both hybridoma 2E3BCMGbcl-2 and mock transfectant 2E3BCMG were inoculated in DME medium supplemented with 9% serum, and cultured with or without additional 2% serum feed at day 4. 2E3BCMGbcl-2: (●) no additional feed, (■) with additional feed; 2E3BCMG: (○) no additional feed, (□) with additional feed.

Figure 6. Antibody production by fed-batch culture of bcl-2 overexpressing hybridoma. Both hybridoma 2E3BCMGbcl-2 and mock transfectant 2E3BCMG were cultured as described for Fig. 5. Antibody concentration in culture supernatant was determined by ELISA. 2E3BCMGbcl-2: (●) no additional feed, (■) with additional feed; 2E3BCMG: (○) no additional feed, (□) with additional feed.

2E3, there was no significant difference in growth rate, viability, and antibody production (data not shown).

Sensitivity to serum limitation

Both the bcl-2 overexpressing hybridoma 2E3BCMGbcl-2 and the control cell lines were cultured at low serum concentration in the medium. At 0.5% serum concentration, the culture of 2E3BCMGbcl-2 could maintain viable cell density higher than the initial cell density for almost five days, while that of the control could for only one day (Fig. 3). However, both of 2E3BCMGbcl-2 and the control died out in one day when cultured without serum. These results suggested that bcl-2 required minor amounts of some serum components to suppress apoptosis of the cells effectively.

Sensitivity to glutamine limitation

The bcl-2 overexpressing hybridoma 2E3BCMGbcl-2 and the control cell lines were cultured with or without glutamine in the medium supplemented with 9%

serum dialyzed to remove glutamine. The bcl-2 overexpressing hybridoma could survive two days longer than the control at 0 mM glutamine (Fig. 4). This result indicated that apoptosis of the hybridoma was less sensitive to glutamine limitation than to that of serum. In the medium supplemented with 2 mM glutamine, the control grew as fast as until day 2 of the culture and after that slower than 2E3BCMGbcl-2 cells, and then started dying at day 3 (Fig. 4). Since we inoculated the cells at a low cell density to avoid apoptosis caused by limitation of some components other than glutamine, by accumulation of toxic metabolites, or by oxygen depletion, we inferred that glutamine was depleted by day 3 of the culture, resulting in the cell death after that. Overexpression of bcl-2 was again effective to improve survival of the cells in this case.

Fed batch culture

Even the bcl-2 over-expressing cells died quickly when completely deprived of serum as reported above. Therefore, we examined the effect of additional serum

feed in the late exponential phase of culture on growth and survival of the cells. The bcl-2 overexpressing hybridoma 2E3BCMgbcl-2 and the control were inoculated in DME medium supplemented with 9% serum and cultured for four days, and then serum was added to the cultures at 2% of the culture volume at day 4. After the serum addition, the hybridomas were cultured for 6 more days. For comparison, both hybridomas were also cultured without additional serum feed. Figs. 5 and 6 show the growth data and the MAb production of the cultures, respectively. For culture of the control cell, the additional serum feed made no significant difference in cell density and antibody production. On the contrary, the additional serum feed at day 4 raised maximum viable cell density by 35% and antibody production by 25% for culture of the bcl-2 overexpressing cells. In comparison with the control cell, the bcl-2 overexpressing cell reached two-fold higher viable cell density and produced three-fold higher antibody levels when both hybridomas were cultured by fed-batch operation.

Conclusions

We demonstrated that culturing bcl-2 overexpressing hybridoma in fed-batch operation is effective to prolong viable culture period and increase viable cell density and antibody production.

Endowing cells resistant to apoptosis by transfecting with bcl-2 gene would make fed-batch operation easier, rendering the timing of additional feed less critical for survival and further growth of cells.

Acknowledgments

We thank Dr. Yoshihide Tsujimoto and Dr. Hajime Karasuyama for the generous gifts of human bcl-2 DNA and vector BCMGSneo, respectively.

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