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Special Issue

Establishing apoptosis resistant cell lines for improving protein productivity of cell culture

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Abstract

The authors established apoptosis resistant COS–1, myeloma, hybridoma, and Friend leukemia cell lines by genetically engineering cells, aiming at more efficient protein production by cell culture. COS–1 cells, which are most widely used for eukariotic gene expression, were transfected with human bcl–2 gene. Both bcl–2 and mock transfected COS–1 cells were cultured at low (0.2%) serum concentration for 9 days. The final viable cell number of the bcl–2 transfected cells was ninefold of that of the mock transfectants. Both bcl–2 and mock transfectants were further transfected with the vector pcDNA-λ containing SV40 ori and immunoglobulin λ gene for transiently expressing λ protein. The bcl–2 expressing COS–1 cells produced more λ protein than the mock transfected COS–1 cells after 4 days posttransfection.

Mouse myeloma p3-X63-Ag.8.653 cells, which are widely used as the partner for preparing hybridoma, and hybridoma 2E3 cells were transfected with human bcl–2 gene. Both bcl–2 transfected myeloma and hybridoma survived longer than the corresponding original cells in batch culture. The bcl–2 transfected 2E3 cells survived 2 to 4 four days longer in culture, producing 1.5- to 4-fold amount of antibody in comparison with the mock transfectants.

Coexpression of bag–1 with bcl–2 improved survival of hybridoma 2E3 cells more than bcl–2 expression alone. The bag–1 and bcl–2 coexpressing cells produced more IgG than the the cells expressing bcl–2 alone.

Apoptosis of Friend murine erythroleukemia(F-MEL) cells was suppressed with antisense c-jun expression. The antisense c-jun expressing cells survived 16 days at non-growth state.

Introduction

Various useful proteins such as cytokines, vaccines, and antibodies are produced by culture of mammalian cells. Production of the proteins of each batch of culture increases when the viable culture period, that is, the growth and viable non-growth culture period extends (Suzuki and Ollis, 1990; Takahashi *et al*., 1994).

However, the cells tend to die quickly after reaching the maximum cell density and hence the viable non-growth culture period is short as two to three days (Duval *et al*., 1990; Vomastek and Franěk, 1993). Therefore, preventing the cells from death which starts in the late exponential growth phase (Vomastek and Franěk, 1993) and maintaining them viable in batch culture for a longer time period should increase protein production of the culture as schematically shown in Figure 1. The 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

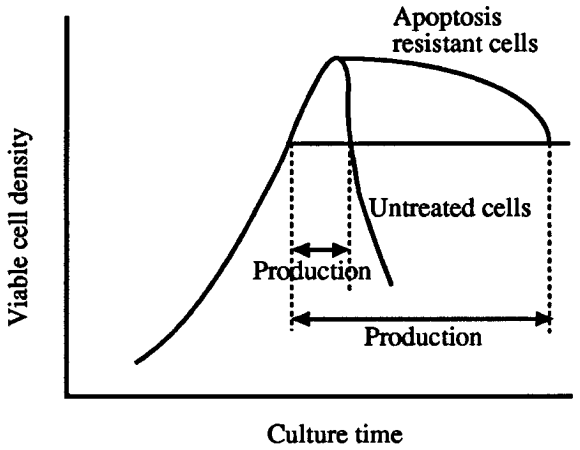

Figure 1. Conceptual drawing of improvement of cell culture productivity by genetically engineering cells to be apoptosis resistant.

culture. In general, cell death may follow two distinct patterns: necrosis and apoptosis, a mechanism of programmed cell death. Reportedly, the cell death in the late exponential growth and stationary phases of batch culture is mostly apoptosis (Mercille and Massie, 1994). Therefore, establishing apoptosis resistant cell lines for protein production should improve the protein productivity of cell culture.

Recently we genetically engineered several cell lines used for protein production to be resistant to apoptosis: bcl–2, an apoptosis suppressive gene, was overexpressed in COS–1 cells, myeloma cells, and hybridoma cells; bcl–2 was coexpressed in hybridoma cells with bag–1 which was known to suppress apoptosis synergistically with bcl–2 (Takayama *et al*., 1995); and antisense c-jun gene expression was induced to suppress c-jun gene, a presumed apoptosis inducing gene, in Friend murine erythroleukemia (F-MEL) cells. These modified cells survived at the non-growth state significantly longer than the corresponding original cell lines. These apoptosis resistant cells produced fourfold amount of the proteins at maximum per batch culture in comparison with the original cells. We report the features of these newly established apoptosis resistant cell lines.

Overexpession of bcl–2 in COS–1 cells

Transient expression in COS–1 cells is the most widely used of all eukariotic expression systems. Therefore, development of apoptosis resistant COS–1 cell line will contribute to improvement of research efficiency. Over the course of a transfection experiment, COS–1 cells accumulate over 10^5 copies per cell of recombinant expression plasmids containing the SV40 origin of replication (Mellon *et al*., 1981) and express high levels of foreign DNA sequences. Expression in this system is transient because replication of the transfected plasmids continues unchecked until the cells die (at 70–90 h posttransfection), presumably because they can not tolerate the high levels of extrachromosomally replicating DNA. Although we have not yet strictly confirmed whether this cell death is apoptosis, we observed the DNA ladder pattern, which is typical for apoptotic cells, in the electrophoresced gel of the DNA extract from COS–1 cells transiently expressing immunoglobulin λ. Therefore, assuming that COS–1 cells apoptotically die during transient expression of foreign proteins, we transfected COS–1 cells with human bcl–2 gene which is known to suppress apoptosis (Vaux *et al*., 1988; Nunez *et al*., 1990).

Materials and methods

The human bcl–2 cDNA fragment was obtained as a 0.95-kb EcoRI fragment from plasmid pCΔj-bcl–2 (Tsujimoto, 1989) and inserted into the EcoRI site of vector Bluescript II KS+ (Stratagene). Properly oriented XhoI-NotI fragment including the bcl–2 cDNA of this construct was inserted into the corresponding sites of expression vector BCMGSneo (Karasuyama *et al*., 1990). The transcription of bcl–2 cDNA sequences is driven by CMV promoter. BCMGSneo has 69% region of BPV–1, which makes the vector amplifiable as episomes in transfected cells (Karasuyama *et al*., 1990). Hence, high expression of the Bcl–2 protein could be expected owing to the strong CMV promoter and the high copy number of BCMGSneo-bcl–2 vector in the cells.

The cells were transfected with the DNA constructs by electroporation using a self-made apparatus. Stable transfectants were selected in the presence of G418 (Gibco) at 1 mg ml^{-1}. Mock transfectants used as control were similarly prepared using BCMGSneo.

A vector pcDNA-λ to be used for transiently expressing immunoglobulin λ chain as a model protein to be produced by COS–1 cells was constructed by inserting cDNA of mouse λ chain into pcDNA3 vector (Invitrogen) that has SV40 ori and cytomegarovirus promoter.

pcDNA-λ was introduced into COS–1 cells by deae-dextran method.

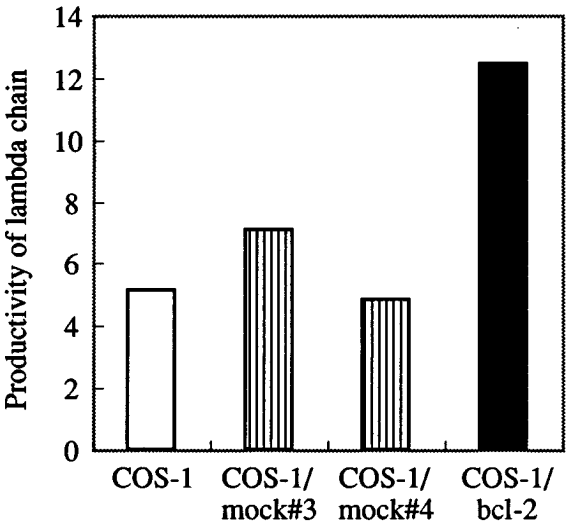

Figure 2. Improved productivity by apoptosis resistant COS–1 cells. The amounts of λ chain produced by 14 days posttransfection relative to those by 4 days posttransfection are shown for original COS–1 cells, human bcl–2 expressing COS–1, and two mock transfectants cultured in DMEM supplemented with serum (FBS) at 10%.

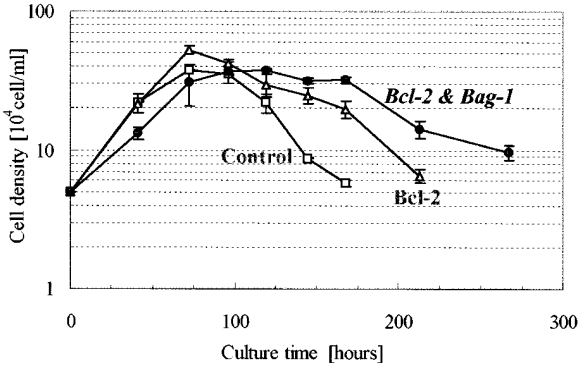

Figure 3. Survival of apoptosis resistant hybridoma cells. The bag–1/bcl–2 coexpressing hybridoma 2E3 cells, the bcl–2 expressing 2E3, and the untransfected 2E3 were cultured respectively in DMEM supplemented with serum (FBS) at 10% for 11 days.

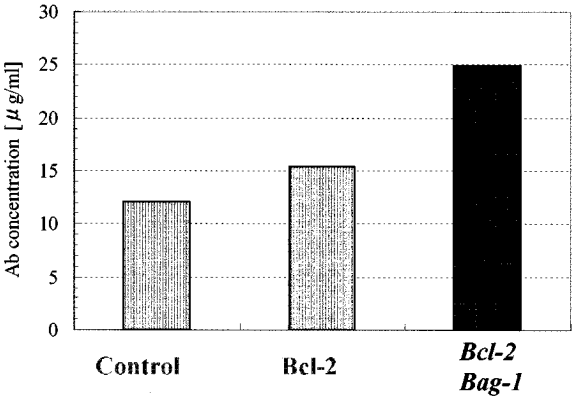

Figure 4. Antibody production by the apoptosis resistant hybridoma cells. Antibody concentration in the culture medium was determined at the last day of the each culture whose viable cell density is shown in Figure 3.

Results

The high expression of human Bcl–2 protein in the bcl–2 transfectants was confirmed by western blotting (data not shown). Both bcl–2 and mock transfectants were cultured for 9 days in low serum (0.2%) culture medium for comparison of survival of both cell lines. The final viable cell number of the bcl–2 transfectants was ninefold of that of the mock transfectants. An example of λ chain production by the bcl–2 transfectants, the mock transfectants, and the original COS–1 cells is shown in Figure 2. The bcl–2 transfectants produced more λ chain than the mock transfectants and the original COS–1 cells after 4 days posttransfection.

Overexpression of bcl–2 in hybridomas and myeloma

We transfected hybridoma 2E3 (Makishima *et al.*, 1992) cells with human bcl–2 gene by the method described above. As we reported elsewhere (Itoh *et al.*, 1995), overexpression of bcl–2 in hybridoma 2E3 cells delayed the initiation of apoptosis, and prolonged viable culture period 2 to 4 days, depending on culture conditions. The bcl–2 transfectants produced 1.5- to 4-fold amount of antibody per batch culture in comparison with the mock transfectants. An example of time course profile of viable cell concentrations of the cultures of the bcl–2 transfectants and the original 2E3 cells is shown in Figure 3. In this case, the bcl–2 transfectants produced 1.5-fold IgG in comparison with the original 2E3 cells as shown in Figure 4, although they did 4-fold in another case (Itoh *et al.*, 1995).

Overexpression of human bcl–2 in mouse myeloma p3-X63-Ag.8.653 also resulted in improved survival, though less remarkable than the case of hybridoma 2E3 (data not shown).

Coexpression of bag–1 with bcl–2 in hybridoma cells

BAG–1 is a Bcl–2 binding protein, and coexpression of bag–1 and bcl–2 provided markedly increased protection from cell death of a human lymphoid cell line, Jurkat (Takayama *et al*., 1995). We tried to make hybridoma 2E3 resistant to apoptosis by coexpression of bag–1 with bcl–2 for improving antibody productivity of the hybridoma culture.

The bag–1 cDNA was inserted in pZeoSV vector (Invitrogen), and then introduced by electroporation into 2E3 cells expressing the human bcl–2. The cells coexpressing bag–1 and bcl–2 were selected by culturing the transfected cells in medium containing Zeocin and G418.

The expression of bag–1 in the selected cells was confirmed by westernblotting. Original 2E3 cells, the bcl–2 transfectants, and the bag–1 and bcl–2 coexpressing cells were cultured respectively for 11 days. The resulting improvements of the survival and the protein production were shown in Figures 3 and 4, respectively. Coexpression of bag–1 and bcl–2 prolonged viable culture period by 4 days and by 1 day, increasing the antibody production of the culture, in comparison with the cultures of original 2E3 cells and the cells expressing bcl–2 alone, respectively.

Suppression of c-jun in FMEL cells

C-jun protein is a proto-oncogene to play important roles in cellular proliferation. Inhibition of c-jun expression may suppress cell growth. Recently it is speculated that c-jun protein functions also as an inducer of apoptosis. Therefore, we hypothesized that inhibition of c-jun expression can suppress overgrowth and apoptosis of cells at the same time. We suppressed c-jun using antisense c-jun gene whose expression was designed to be controlled by dexamethasone (DEX) inducible MMTV promoter.

Materials and methods

F-MEL cells were supplied by Riken Cell Bank. pMS-G c-jun AS (antisense) dhfr vector was gift from E. V. Prochownik (Smith and Prochownik, 1992). The vector includes G418 resistance gene. The anti-sense c-jun gene is located downstream MMTV promoter. The cells were transfected with pMS-G c-jun AS dhfr, selected by culturing in DMEM medium supplemented with G418. c-jun AS dhfr gene was amplified by culturing the transfected cells in the medium supplemented with methotrexate (MTX) for several months. The obtained cells were called ASjun cells.

Results

mRNAs were extracted from the cells and westernblotted. c-jun mRNA was not detected for ASjun cells cultured with DEX, while detected for ASjun cells cultured without DEX (data not shown). ASjun cells cultured with DEX were able to maintain the viability at higher than 85% for 16 days while the original F-MEL cells were only for 4 days (Figure 5).

Discussion

All the methods we reported above, the antisense c-jun expression, the bcl–2 overexpression, and the bag–1 and bcl–2 coexpression, could improve survival of cell lines used for protein production. These apoptosis resistant cell lines are easy to culture. For example, they can grow with less serum, and do not easily die out even if the medium exchange is delayed. Effect of using apoptosis resistant cells on protein productivity is variable culture by culture, for example, 1.5-fold, 2-fold, or 4-fold improvement, owing to still unknown mechanisms. A probable explanation is the following. The cells stop growth in unfavorable culture conditions caused by overgrowth, and almost simultaneously start dying by apoptosis. The growth limiting condition such as amino acid or glucose limitation inhibits protein synthesis; hence, although the apoptosis resistant cells can survive, they can not synthesize protein. On the other hand, the growth limiting condition such as serum limitation does not directly inhibit protein synthesis; hence, the survived cells can continue to produce the protein, resulting in improved protein productivity.

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