Special Issue

Reinforcing apoptosis-resistance of COS and myeloma cells by transfecting with bcl-2 gene

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Abstract

COS, myeloma and HeLa cells, which are commonly used for protein production by cell culture, were transfected with human bcl-2 gene encoded on the shuttle vector BCMGS. Expression of human bcl-2 improved survival of cells remarkably, mildly, or negligibly for COS, myeloma, and HeLa, respectively. Four clones were obtained from the human bcl-2 expressing cell population of COS cells. They expressed human bcl-2 almost at the same level. The viable cell numbers were 6, 2.5, 2.5, and 0.8 times as many for the clones #8, #5, #6, and #7, respectively, as for the control COS cells, when they were cultured at low (0.2%) serum concentration for 9 days. The bcl-2 overexpressing COS cells showed morphology different from that of the control COS cells in serum limited condition. When transfected with mouse lambda protein gene carried by an SV40-derived vector, clone #8 of the bcl-2 transfected COS cells continued the transient expression of lambda protein longer than the control COS cells.

Introduction

Mammalian cell culture is indispensable for production of proteins that require posttranslational modifications for exhibiting their native activities. But mammalian cells are fastidiously sensitive to culture conditions and they tend to trigger the suicide program encoded on their own chromosome when they are exposed to unfavorable culture conditions, causing the 'fin' of the culture. This suicide named apoptosis is caused by many intercellular and intracellular conditions such as starvation of growth factors or serum components, excess shearing force due to agitation or bubbling, accumulation of toxic metabolites secreted by cells, UV or gamma irradiation, heat shock, and viral infection (Williams *et al.*, 1993; Cotter *et al.*, 1995). Modifying the cell lines which have been widely used for protein production to reinforce their apoptosis-resistance will contribute to improvement of the efficiency of protein production by mammalian cell culture. For example, in production of vaccine or viral vectors for genetic ther-

apy, culturing cells free of serum components is desirable for avoiding biological contamination with virus or mycoplasma in the serum; therefore, it is important to establish cell lines that are viable in culture free of serum components. For another example, cells that can survive longer at late batch culture, where nutrients are depleted and toxic metabolites are accumulating, would produce more products per batch of culture. According to this concept the author group converted a hybridoma cell line into apoptosis resistant one, by transfecting with anti-apoptotic gene bcl-2. The hybridoma cells overexpressing Bcl-2 produced monoclonal antibody in a batch culture four times as much as the untransfected ones (Itoh *et al.*, 1995; Suzuki *et al.*, 1997).

The mechanism of apoptosis has not been fully elucidated; several cellular components having roles in apoptosis were reported; among them, Bcl-2 is notable. The bcl-2 gene was first identified by Tsujimoto *et al.* (Tsujimoto *et al.*, 1985) and its function was elucidated by Vaux *et al.* (Vaux *et al.*, 1988).

It has been reported that overexpression of Bcl-2 in many cells suppressed or delayed apoptosis induced by various causes, although details of Bcl-2 function have been unknown yet. In this study, we transfected COS-1, myeloma X63.653, and HeLa cell lines with human bcl-2 gene to make them apoptosis-resistant for improving protein productivity of the cell culture.

In transient expression with COS cells, vectors derived from SV40 are highly amplified up to 10^6 copies in the nucleus and then proteins encoded on these vectors can be expressed intensively (Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 1995; Mellon *et al.*, 1981). This system is well established and available, therefore many laboratories use it for rapid preparation of various recombinant proteins expressed in mammalian cells. In addition, it was recently reported that the transient expression system was applicable for large scale protein production (Blasey *et al.*, 1996). However, COS cells start dying after the transfection with SV40 vectors. Although the mechanism of this cell death and its type, apoptosis or necrosis, have been unknown yet, this cell death might be one of the cases that viral DNAs are highly amplified, inducing apoptosis of host cells, since SV40 origin vectors are amplified too much in the nucleus of COS cells.

Along this concept, we previously transfected COS cells with human bcl-2 gene to make them apoptosis-resistant, and reported that the uncloned population of COS-1 cells overexpressing human Bcl-2 produced the protein encoded on SV40 vector more than twice as much as the control COS-1 cells (Fujita *et al.*, 1996). In the present work, we cloned the bcl-2 transfected COS-1 cells, and studied the apoptosis-resistance, the expression level of Bcl-2, and the prolonged protein production period of the culture.

HeLa cells can be cultured in both suspended and adhering conditions, which seems to be advantageous for protein production by cell culture.

Myeloma cells are widely used for establishing hybridoma cells for producing monoclonal antibody. Since they grow fast, and are easy to culture, they are often used for recombinant protein production as well.

Converted into apoptosis-resistant cells by transfecting with bcl-2 gene, COS, HeLa and myeloma cell lines are expected to be better applicable for production of various recombinant proteins by cell culture.

Materials and methods

Cell culture

COS-1 cells were cultured in Dulbecco's modified Eagle's (DME) medium (Nissui, Tokyo) supplemented with 10% (v/v) calf serum (CS), 20 mm N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES), 0.2% NaHCO₃, 2 mm glutamine and 0.06 mg ml⁻¹ kanamycin. The cells were grown in $\varnothing$ 100 mm dish at 37 °C in humidified air containing 5% CO₂. To remove adhering cells from the dish, culture medium was replaced by 500 μ l of 0.02% (w/v) trypsin and 0.05% (w/v) EDTA in PBS, and then the cells were incubated at 37 °C for 5 min. HeLa cells were cultured as COS-1 cells except for using fetal bovine serum (FBS) instead of CS. Myeloma X63.653 cells are suspension-cultured in DME medium supplemented with 10% FBS using the flask or dishes.

Construction of Bcl-2 expression plasmids

p Δ j-bcl-2 (Tsujimoto *et al.*, 1985) includes 0.95 kbp bcl-2 ORF between EcoRI restriction enzyme sites. Its fragment was ligated into EcoRI site in pBluescript II KS+ (Stratagene). The properly oriented fragment of bcl-2 cDNA between XhoI and NotI sites was inserted downstream of the CMV promoter in BCMGS-neo or -hygro (Karasuyama *et al.*, 1988; 1990), yielding a vector termed BCMG-bcl2-neo or BCMG-bcl2-hygro (Figure 1).

Transfection and selection

BCMG-bcl2-neo was transfected into COS-1 and HeLa cells by electroporation, respectively, and so was BCMG-bcl2-hygro into X63.653 cells. The mock transfected control cells were prepared similarly except for using BCMGS-neo or -hygro. Cells were electroporated with a hand-made apparatus, and selected by 400 μ g ml⁻¹ of G418 or 1 mg ml⁻¹ of hygromycin.

Western blotting

The 10^5 cells were harvested and lysed in 100 μ l of 10 mm TrisHCl (pH 7.4), 1% Triton X-100, 0.15 mm NaCl, 50 mg ml⁻¹ phenyl methanesulfonyl fluoride (PMSF), and 2 μ g ml⁻¹ aprotinin at 4 °C for 30 min. After debris was eliminated by centrifuge, the lysate was analyzed by Western blotting using mouse anti human Bcl-2 monoclonal antibody (Boehringer Man-

Figure 1. Construction of Bcl-2 expression vector. The *bcl-2* cDNA was ligated downstream of the CMV promoter. BPV 69% region works to multiply the vector in the nucleus.

neheim) and goat anti mouse Ig polyclonal antibody conjugated with horse radish peroxidase (HRP) (Bio Source International, Inc. Tago Products). Human Bcl-2 bands were detected by ECL kit (Amersham).

Estimation of apoptosis resistance

The *bcl-2* transfected COS-1 and HeLa cells were inoculated at 10^5 cells ml^{-1} and incubated in DME medium supplemented with 0.2% or free of FBS, respectively, in $\varnothing$ 60 mm dishes. The *bcl-2* transfected myeloma cells were inoculated at 7×10^4 cells ml^{-1} in the 24 well plate and incubated in DME medium supplemented with 0.2% FBS. After various incubation periods, cells were collected, washed twice with PBS and stained with trypan blue, and viable cells were counted with hemocytometer.

Cloning apoptosis-resistant cells

The *bcl-2* transfected COS-1 cells were limiting-diluted in each 100 μl well of a 96-well plate containing complete medium supplemented with 400 μg ml^{-1} of G418, and incubated for a few weeks. Then each growing colony was harvested as a clone.

Examination of protein productivity

Mouse lambda chain cDNA was amplified by PCR and inserted between EcoRI and NotI sites on plasmid pcDNA3 (Novagen), yielding a plasmid termed pcDNA- λ . For expressing lambda chain transiently, 10 μg of the plasmid was transfected into 5×10^5 cells in $\varnothing$ 100 mm dishes with DEAE-dextran/chloroquine method (Current Protocols in Molecular Biology, John Wiley & Sons, Inc, 1995). After the transfection for transient expression, the cells were cultured in 10%

Figure 2a. Western blot analysis of expressed human Bcl-2. The lysates of the bcl-2 transfectants and the control or wild type cells were electrophoresed. Bcl-2 bands were detected using mouse anti human Bcl-2 monoclonal antibody as described in the section Materials and Methods. A: COS-1/bcl-2 and COS-1/cont, B: X63.653/bcl-2 and X63.653/cont, C: HeLa/bcl-2 and wild type HeLa, D: COS-1/bcl-2 clones.

Figure 2b.

serum medium, and the culture supernatant was sampled. The concentration of lambda protein in the samples was measured by enzyme-linked immunosorbent assay (ELISA) using rabbit anti mouse lambda antisera (Organon Teknica Corp.) and goat anti mouse lambda antibodies conjugated with HRP (Southern Biotechnology Associates, Inc.).

Results

Cell engineering to establish Bcl-2 overexpressing cell lines

COS-1 cells were transfected with BCMG-bcl2-neo and BCMGS-neo, and termed COS-1/bcl-2 and COS-1/cont, respectively. Similarly, HeLa/bcl-2 and HeLa/cont were prepared. Myeloma X63.653 cells were transfected with BCMG-bcl2-hygro and BCMGS-hygro, and termed X63.653/bcl-2 and X63.653/cont, respectively. The vector carrying the

gene that provides resistance to hygromycin instead of neomycin as a selection marker was applied to the myeloma, since a neomycin resistant vector was planned to be used for expressing a protein in X63.653/bcl-2 cells.

BCMGS- vectors were derived from bovine papilloma virus type-1 (BPV-1) and can multiply 20–100 copies in the nucleus (Karasuyama *et al.*, 1988; 1990). Hence the transfected bcl-2 cDNA is expected to be overexpressed owing to both of the strong CMV promoter and the high copy number of the vectors in the nucleus. For detection of the expressed Bcl-2 protein, Western blot analysis was performed for the cells selected by neomycin or hygromycin resistance. The lysates of COS-1/bcl-2, X63.653/bcl-2, and HeLa/bcl-2 as well as their controls, COS-1/cont, X63.653/cont, and HeLa/cont were applied to each lane of electrophoresis gel, respectively. The bands indicating Bcl-2 protein at 26 kDa were visible for the bcl-2 transfectants, but not for the controls (Figures 2a, b, c). Contrary to our expectation, HeLa expressed Bcl-2 pro-

Figure 2c.

tein weakly in comparison with the other bcl-2 transfectants (Figure 2c). There was no difference in cell morphology between the bcl-2 transfectants and the control cells. But it was observed through microscope that COS-1/bcl-2 stayed viably longer than the control when growth-arrested by contact inhibition; COS-1/cont began piling up, and then dyed floating into culture supernatant under the contact inhibition, while COS-1/bcl-2 stayed as a single flat layer of viable cells at the bottom of the dish.

Apoptosis resistance against serum starvation

To examine whether the cells overexpressing human Bcl-2 acquired apoptosis resistance, we cultured the bcl-2 transfectants and the control cells in the medium of low serum concentration, and examined the number of viable cells. Reportedly, cultured cells under a serum-limited condition induce apoptosis, which is a commonly used method of evaluating apoptosis resistance (Henderson *et al.*, 1991; Tsujimoto *et al.*, 1989a; 1989b). The viable cell number of COS-1/cont that was incubated in the medium containing 0.2% serum gradually decreased to the half of the initial one on day 9. In the same culture condition, that of COS-1/bcl-2

slightly decreased in 2 days (data not shown) and then increased to 4-fold of the initial one on day 9 (Figure 3, uncloned).

X63.653/bcl-2 cells could proliferate for two days at low (0.2%) serum concentration, and then started dying slowly, while X63.653/cont cells did not proliferate at all, dying quick after day 3 of the culture (Figure 4). The viable cell number of X63.653/bcl-2 was 2-fold that of the X63.653/cont on day 6 of the culture. Although human Bcl-2 was highly expressed in X63.653/bcl-2 cells as the Western blot indicated (Figure 2b), the effect of Bcl-2 expression on survival of the myeloma was less remarkable than that of COS-1.

HeLa/bcl-2 expressed Bcl-2 weakly in comparison with COS/bcl-2 and X63.653/bcl-2 as the Western blot indicated (Figure 2c). Probably because of this low expression level of Bcl-2, HeLa/bcl-2 did not exhibit any improvement of survival while cultured in the serum free medium.

Cloning apoptosis-resistant COS-1

To establish an apoptosis-resistant COS-1 cell line, the COS-1/bcl-2 cells were cloned, and the apoptosis resistance of the each clone was examined in the low serum condition. The survival and growth of COS-1/bcl-2 in the low (0.2%) serum medium varied from clone to clone (Figure 3). Clone #8 grew and survived best to 6-fold the viable cell number of the control on day 9, clone #5 and #6 did to 2-fold, and clone #7 did to 0.8-fold (Figure 3), although the each clone expressed human Bcl-2 almost at the same level as the western blot indicated (Figure 2d). At the low serum concentration, all the COS-1/bcl-2 clones including clone #7, of which viable cell number was lower than that of COS-1/cont, looked similar in shape to those cultured with 10% FBS, while COS-1/cont did not form stable shape in the low serum medium (observation through microscope). Clone #8 was assigned to the following study as a newly established apoptosis-resistant COS-1 cell line.

Protein productivity

In transient expression using COS-1 cells, the cells are transfected with SV40 vector encoding gene for protein, then start dying while producing the protein at a high rate. In several days after the transfection, the protein production ceases due to the cell death. We examined whether the production period can be prolonged by improving survival of COS-1 cells, applying

Figure 2d.

Figure 3. The viable cell number of COS-1 cells cultured in low serum medium. 10^5 cells of the each COS-1/bcl-2 clone, the uncloned COS-1/bcl-2, and COS-1/cont were cultured, respectively, in the medium supplemented with 0.2% FBS. After 9 days of incubation, the cells were harvested and counted by trypan blue exclusion. #0: COS-1/bcl-2 before cloning, #5, #6, #7, and #8: clones of COS-1/bcl-2. All data represent 3 replicated experiments.

Figure 4. The viable cell number of myeloma cells cultured in low serum medium. 7×10^4 cells of X63.653/bcl-2 and X63.653/cont were cultured, respectively, in the medium supplemented with 0.2% FBS. The viable cells were counted by trypan blue exclusion.

the newly established apoptosis-resistant COS-1 cell line, COS-1/bcl-2 clone #8.

For comparing protein productivity of COS-1/bcl-2 and COS-1/cont, we used mouse lambda protein as a model protein product. As this protein is stable in culture medium and secreted to culture medium when expressed in mammalian cells, daily protein production can be easily determined by measuring the concentration of the protein in culture supernatant. After transfection with the SV40 vector carrying the lambda protein gene for transient expression, clone #8 cells and COS-1/cont cells were incubated in complete medium, and the culture supernatant was sampled on day 4, 6, 8, 10, 12 and 14 posttransfection. The concentration of lambda protein in each sample was measured by ELISA. The lambda protein concentration was shown in Figure 5 as the ratio to that on day 6 instead of the absolute concentration, since it is often influenced by transfection efficiency, which usually varies run by run, and can not be stabilized. It is usually recommended that secreted protein products should be harvested on day 6 (Current Protocols in Molecular Biology, John

Wiley & Sons, Inc, 1995), since protein production seems to stop on day 6. In our experiment, COS-1/cont almost stopped protein production on day 6 posttransfection, but COS-1/bcl-2 clone #8 seemed to continue production till day 10 or 12 posttransfection (Figure 5). The experiment with untransfected COS-1 cells yielded the results similar to that with COS-1/cont. In this experiment, we used 10% serum medium because it seemed to be a common practice for culturing COS cells to produce protein as described in Current Protocols. Low serum culture, however, may give larger ratio of protein productivity of COS-1/bcl-2 to COS-1/cont, since COS-1/bcl-2 clone #8 but not COS-1/cont proliferates even in low serum medium (Figure 3). The result shown in Figure 5 suggested that overexpression of Bcl-2 suppressed cell death at late posttransfection, prolonging production period by 4 to 6 days.

Figure 5. Lambda protein production at late posttransfection by COS-1/bcl-2 clone #8 and COS-1/cont. Mouse lambda protein was produced by transient expression as described in the section Materials and Methods. Each 5×10^5 cells of COS-1/bcl-2 and COS-1/cont were transfected with $10 \mu\text{g}$ of the SV40 vectors carrying lambda gene, and cultured in the medium supplemented with 10% CS. The culture supernatant was sampled on day 4, 6, 8, 10, 12 and 14 posttransfection. The concentration of lambda protein in the culture supernatant was determined by ELISA and normalized to that of the respective culture on day 6.

Discussion

Method of overexpressing bcl-2

For acquiring remarkable apoptosis resistance, target cells usually should overexpress bcl-2 gene. In this study, we chose BCMGS- shuttle vector system, which is featured by its high multiplication in the nucleus and its powerful CMV promoter, for overexpressing bcl-2 gene; the system successfully worked for COS-1 and myeloma cells in this work, and for hybridoma 2E3O in our previous work. However, it did not work satisfactorily for HeLa cells. HeLa/bcl-2 expressed only little amount of Bcl-2 protein and probably because of the low level expression of Bcl-2, HeLa/bcl-2 did not survive longer than the control cells.

Some more efficient expression system for HeLa cells should be investigated.

Expression level of Bcl-2 did not always correlate with survival

Although the clone #3 to #8 of COS-1/bcl-2 expressed Bcl-2 at almost the equal level, there was much difference among them in survival at the low serum concentration, the reason of which is unknown.

X63.653/bcl-2 expressed Bcl-2 at the high level. However, there was only moderate difference of survival between the bcl-2 transfectant and the control in the low serum condition. Recently, it was reported that another myeloma NSO endogenously expressed Bcl-XL, which is known as a bcl-2 alternative, at a high level, and hence the overexpression of bcl-2 did not improve survival of NSO cells (Gauthier *et al.*, 1996; Murray *et al.*, 1996). Hence, the expression level of endogenous Bcl-XL in X63.653 cells should be examined. One of probable methods of improving apoptosis

resistance of the myeloma cells is the application of some other functional factors which work via mechanisms different from Bcl-2; Bcl-2 has negative role in preparation of active ICE like enzymes from their precursors; the enzymes are thought to be main mediators of apoptosis process. The proteins encoded in cowpox virus crmA and baculovirus p35 genes inhibit enzymatic activity of ICE like proteases, suppressing apoptosis via mechanisms different from Bcl-2 (Miura *et al.*, 1993; Xue *et al.*, 1995).

Criteria for selecting clone

In this work, we selected clones using the criterion, survival at the low serum concentration. The prolonged protein production by COS-1/bcl-2 clone #8 shown in Figure 5 suggested that the clone survived longer than the control during the transient expression as well, although some more direct supporting data such as the cell viability is necessary to conclude it.

In case of the bcl-2 transfected hybridoma cells, we found that the clones which were most resistant to apoptosis produced the monoclonal antibody at a rate slower than the control cells (data not shown). Therefore, some better criteria to evaluate the survival and the production rate of single cell at the same time should be explored.

Conclusion

By transfecting with human bcl-2 gene, we established a COS-1 cell line and a myeloma X63.653 cell line of which apoptosis resistance was significantly or a little improved, respectively; however, we could not improve survival of HeLa cell line.

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