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Co-expression of bcl-2 and bag-1, apoptosis suppressing genes, prolonged viable culture period of hybridoma and enhanced antibody production

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Abstract

Human bcl-2 and bag-1 DNA were introduced into mouse hybridoma 2E3- O cells and expressed. The expression of bcl-2 in BCMGneo-bcl2 transfectants was confirmed by ELISA and that of bag-1 in pZeo-bag1 was confirmed by western blotting. In batch cultures, the over-expression of bcl-2 prolonged the culture period by 2 days and co-expression of bcl-2 and bag-1 prolonged the culture period by 3 days. The delayed increase in the dead cell number in culture of the bcl-2 and bag-1 cotransfectant indicated the additional antiapoptosis effect of bcl-2 and bag-1 cotransfection in comparison with the bcl-2 only transfection. The bcl-2 transfectants (2E3O-Bcl2) produced antibody twofold per batch culture in comparison with 2E3-O cells transfected with BCMGSneo (2E3O-Mock). Enhancement of this MoAb production was due to the improved survival of the cells and was not due to stimulation of antibody production rate per cell by Bcl-2 expression. And the bcl-2 and bag-1 co-transfectant (2E3O-Bcl2-BAG1) produced antibody approximately fourfold of 2E3O-Mock per batch culture. Enhancement of this MoAb production was due to the improved survival of the cells and was partly due to stimulation of MoAb production rate per cell in the non-growing phase by the cotransfection. The method to engineer hybridoma cells genetically with bcl-2 and bag-1 for increasing viability and productivity would be widely applied for improving antibody productivity of hybridoma cultures.

Introduction

Monoclonal antibodies (MoAbs), which have been widely used as reagents for assay of various compounds and for clinical diagnosis, are produced mostly by culture of hybridomas. The increasing demand for large quantities of MoAbs requires the improvement of MoAb productivity of hybridoma cell culture. In order to improve MoAb productivity, there would be two distinct strategies. One strategy is enhancement of production rate in unit number of viable cells. Antiproliferation often enhances production rate in unit number of viable cells (Suzuki and Ollis, 1990). And another strategy is increase of the integrated cell population. Because hybridoma cells tend to die quickly after reaching the maximum cell density (Duval et al., 1990; Perreault and Lemieux, 1994), preventing cells from death which starts in the late exponential growth phase (Vomastek and Franek, 1993) and maintaining viable in batch culture for longer period should increase the integrated cell population.

Recently, our understanding of cell death has been advanced. Cell death may follow two distinct patterns: necrosis and apoptosis, a mechanism of programmed cell death. In apoptosis, cells shrink, extruding water, converting the cell body into a highly rigid and relatively stable structure; cellular DNAs are fragmented into 200 base pairs (Tomei, 1991; Duke and Cohen,

1992). In necrosis, cells typically swell and release their cytoplasmic contents to the extracellular fluid by breakdown of the plasma membrane; cellular DNA is degraded nonspecifically (Tomei, 1991).

Though the mechanism of apoptosis has not been fully elucidated, several cellular components functioning in apoptosis were reported. Among them, Bcl-2 is known to suppress multiple forms of apoptosis (Pattersson et al., 1992). High levels of human Bcl-2 protein resulted in the better survival of mouse myeloid cells in the absence of the required growth factor (Tsujimoto, 1989). The high levels of the bcl-2 gene product protected human B cells from a variety of agents giving stress including heat shock, ethanol and methotrexate, and the absence of serum in the medium (Tsujimoto, 1989).

We have already reported that overexpression of Bcl-2 in hybridoma prevented cell death and enhanced MoAb production (Itoh et al., 1995; Terada et al., 1997), and Singh et al. reported that overexpression of Bcl-2 enhance survivability of mammalian cells (Singh et al., 1996), and Simpson et al. also reported that bcl-2 transfection increased MoAb production of hybridoma (Simpson et al., 1997). In this report, we aimed increasing this effect. Recently, BAG-1, Bcl-2 association athagogene 1, protein was discovered and co-expression of bag-1 and bcl-2 increased protection from cell death (Takayama et al., 1995). Cell engineering using these apoptosis suppressing genes would enable hybridoma cells to survive longer and could increase MoAb productivity. The object of the present work is to engineer hybridoma cells for improving their survival in order to increase MoAb production per culture by transfecting the cells with human bcl-2 and murine bag-1. Our results show that the viable culture period was prolonged at least 3 days and the MoAb production per culture was enhanced fourfold for the batch culture.

Materials and methods

Cell line and culture conditions

A cell line 2E3-O employed throughout this study is a mouse hybridoma derived from a mouse myeloma P3X63 AG8U.1 by electric fusion with mouse spleen cells (Makishima et al., 1992).

The hybridoma is a high producer of an IgG1 specific to a trinitrophenyl (TNP)-hapten. The cells were cultured in DME medium (Nissui, Tokyo), supplemented with 10% FBS (vol/vol), 20 mM HEPES, 0.2% $NaHCO_3$, 2 mM glutamine, and 0.06 mg ml^{-1} kanamycin. The cells were grown in 24 well plate (Iwaki glass, Tokyo) at 37 ° in humidified air containing CO_2 at 5%.

Viable and dead cell number was determined by counting in a hemacytometer under a phase contrast microscope using trypan blue exclusion.

Establishment Bcl-2 and BAG-1 co-expressing hybridoma cell line

The murine bag-1 cDNA fragment was obtained as a EcoRI fragment from vector Bluescript-bag-1 (Takayama et al., 1995) and inserted into the EcoRI site of vector pZeoSV(Invitrogen), resulting in the vector pZeo-bag1 (Figure 1).

The vector BCMGneo-bcl2 for expressing human bcl-2 (Tsujimoto, 1989) in the hybridoma cells was prepared as reported elsewhere (Itoh et al., 1995).

The hybridoma cells were transfected with the DNA constructs by electroporation using a self-made apparatus. The number of pulses applied was determined so that about 50% of the treated cells should die in 12 h. Stable transfectants were selected in the presence of G418 (Gibco) at 0.25 mg ml^{-1} and 0.25 mg ml^{-1} Zeocin (Invitrogen).

Determination of the quantity of Bcl-2 protein

The hybridoma cells were lysed and amount of Bcl-2 protein was determined by Bcl-2 ELISA kit (Calbiochem, Cambridge MA). All data are normalized by the value for the population.

Western blot analysis of BAG-1 protein

Single-cell suspensions were lysed in 1% Triton X-100, 0.15 mM NaCl and 10 mM Tris (pH 7.4) with 50 mg ml^{-1} PMSF and 2 mg 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%). Gels were transferred to nitrocellulose filter overnight. Blots were blocked with 5% skim milk for 2 h at room temperature. Murine BAG-1 protein was detected by rabbit anti-murine-BAG-1 protein polyclonal antibody (Santa Cruz Biotechnology) to which peroxidase-conjugated goat anti-rabbit Ig polyclonal antibody (Bio Source International, Inc.-Tago Products) bound. The density of the band was measured by a density meter, model ACD-25DX (ATTO).

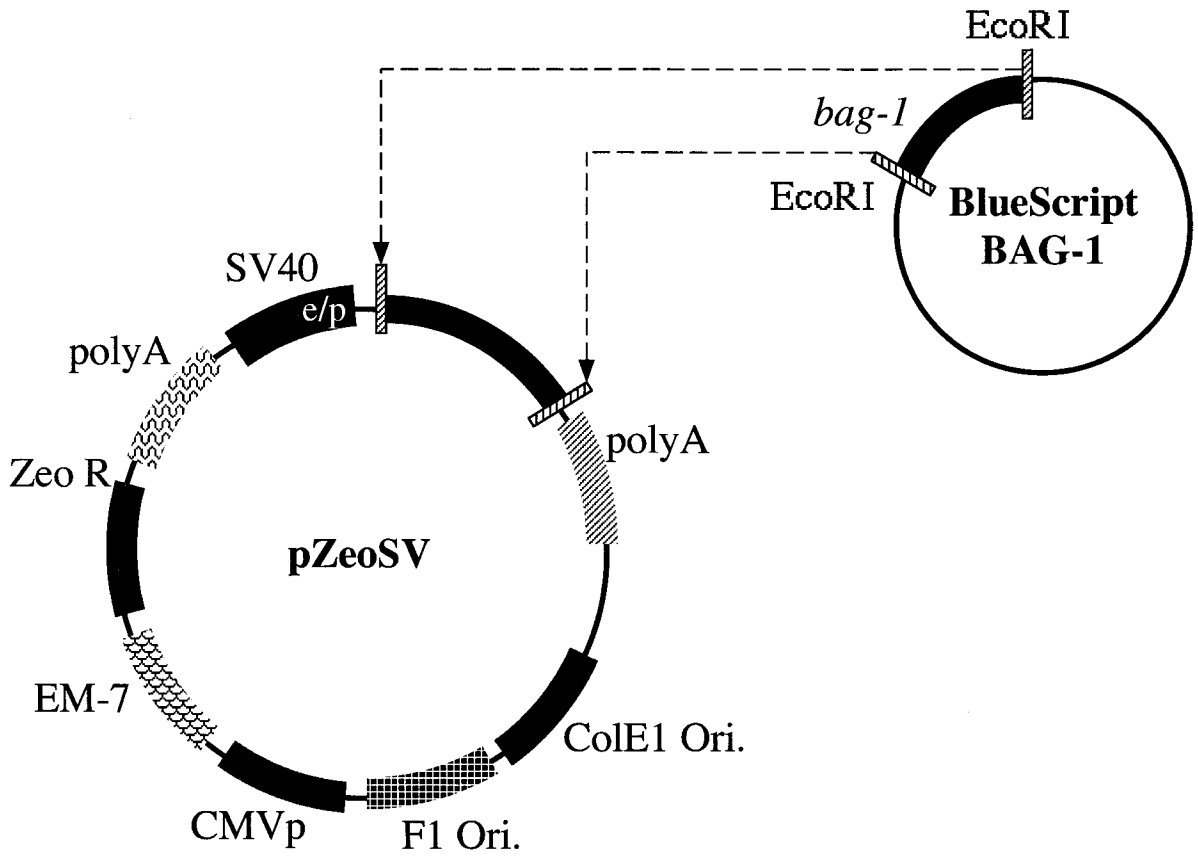

Figure 1. Construction of murine BAG-1 expression vector. The bag-1 cDNA was obtained as a EcoRI fragment from vector Bluescript-bag-1 (Takayama et al., 1995) and inserted into the EcoRI site of vector pZeoSV (Invitrogen).

Determination of antibody concentration

The antibody concentration in the hybridoma culture supernatant was determined by ELISA. MoAb produced by 2E3-O cells was sandwiched by rabbit anti-mouse IgG polyclonal antibody (ZYMED Laboratories) and peroxidase-conjugated goat anti-mouse Ig polyclonal antibody (Bio Source International). The amount of MoAb was determined by measuring absorbance, using o-phenylenediamine dihydrochloride as substrate for peroxidase. The known amount of the purified MoAb secreted by 2E3-O cells was used as standard.

Results and discussion

Cell engineering to establish Bcl-2 and BAG-1 overexpressing cell line

We had already transfected hybridoma 2E3-O cell with human bcl-2 gene and named 2E3O-BCMG-bcl2 (Itoh et al., 1995). In order to generate bcl-2 and bag-1 cotransfectant, we transfected murine bag-1 gene to the bcl-2 transfectant. The murine BAG-1 expression vector pZeo-bag1 was constructed as shown in Figure 1. Then pZeo-bag1 vector and pZeo vector, as a control, were introduced into 2E3O-BCMG-bcl2 cells, respectively. Because pZeo vector contains the zeocyn-resistance gene, the transfectants were cultured in the medium with 250 μM zeocyn for selection of transfectants. By cloning zeocyn-resistant transformants, we obtained 9 clones of pZeo-bag1 transfectant. Then we detected expression of BAG-1 protein in each clone by western blotting. Among these clones, the clone expressing BAG-1 highest was selected and named 2E3O-Bcl2-BAG1. 2E3O-BCMG-bcl2 transfected with pZeo vector was named 2E3O-Bcl2. As negative control, we used 2E3O-BCMGS, transfectant of BCMGS vector, and named it 2E3O-Mock.

We measured expression of BAG-1 protein by

Table 1. Expression of murine BAG-1 protein (29 kDa)

Cell	Density of 29 kDa band	Density of 46 kDa band	Density of 29 kDa band/ Density of 46 kDa band
2E30-Mock	375	413	0.9
2E30-Bcl2	252	332	0.8
2E30-Bcl2-BAG1	519	356	1.5

The densities of the 29 kDa bands supposed to be BAG-1 and those of 46 kDa as a control on Figure 2 were measured by density meter, model ACD-25DX (ATTO).

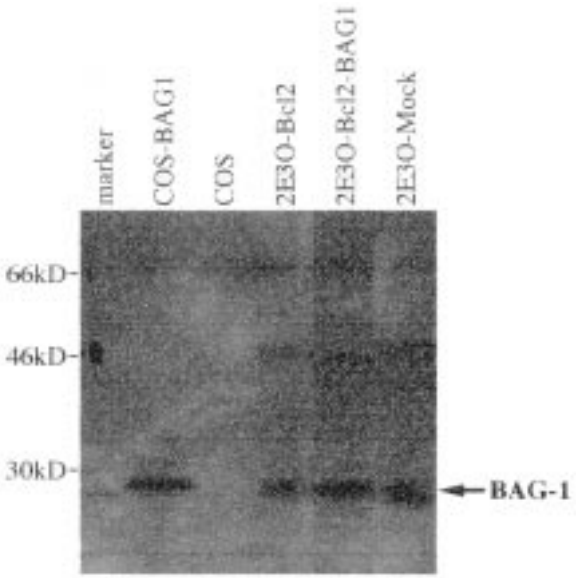

Figure 2. Western blot analysis of the murine BAG-1 protein. The lysate from cells was loaded into each lane. Murine bag-1 protein was detected by rabbit anti-murine-bag-1 polyclonal antibody (Santa Cruz Biotechnology) to which peroxidase-conjugated goat anti-rabbit Ig polyclonal antibody (Bio Source International) bound.

western blotting method (Figure 2). COS-1 cells transfected with pZeo-bag1 was named COS-BAG1 and COS-1 cells transfected with pZeo was named COS. The lysates of COS-BAG1 as positive control, COS as negative control, 2E3O-Mock, 2E3O-Bcl2, and 2E3O-Bcl2-BAG1 were applied to each lane of electrophoresis gel, respectively. The bands indicating BAG-1 protein at 29 kDa were visible for 2E3O-Mock, 2E3O-Bcl2 and 2E3O-Bcl2-BAG1. The densities of the 29 kDa bands were measured with the density meter (Table 1). In order to compare the amount of BAG-1 protein, the density of 29 kDa bands, supposed to be BAG-1, were divided by that of 46 kDa bands and the quotients were shown in Table 1. The normalized 29 kDa band in the lane of 2E3O-Bcl2-BAG1 was nearly twice denser than those of 2E3O-Mock and 2E3O-Bcl2. Jointly with the fact that the bag-1 gene was derived from mouse cells and 2E3-O is a mouse hybridoma, the result of the western blotting would indicate that 2E3-O hybridoma cells synthesize BAG-1 protein, and the bag-1 gene transfection enhanced the BAG-1 synthesis.

Expression level of bcl-2 gene

As we reported previously (Terada et al., 1997; Terada et al., 1998), higher expression of bcl-2 causes more resistant to cell death. In order to estimate effect of bag-1 expression independently of bcl-2 expression level, we compared bcl-2 protein expression of 2E3O-Bcl2-BAG1 to that of 2E3O-Bcl2. Amount of Bcl-2 protein was determined by Bcl-2 ELISA kit (Figure 3). In the lysate from 2E3O-Mock, Bcl-2 protein was not detected. And amount of Bcl-2 protein in the lysate from 2E3O-Bcl2-BAG1 was as much as in the lysate from 2E3O-Bcl2. Therefore, 2E3O-Bcl2 cell is a suitable 'bcl-2 (+) bag-1(–) control' to 2E3O-Bcl2-BAG1 cells.

Effect of Bcl-2 and BAG-1 on cell growth and cell death

Effects of Bcl-2 and BAG-1 on cell population were examined. For investigating whether the expression of Bcl-2 and BAG-1 altered the growth characteristics, 2E3O-Bcl2-BAG1, 2E3O-Bcl2, and 2E3O-Mock were batch-cultured. Both of the bcl-2 and bag-1 transfectant and the bcl-2 transfectant grew at almost the same rate until day 2 during the exponential growth phase, as shown by the total cell numbers in Figure 4a. However, the mock transfectant started dying and the viability fell away to less than 75% before day 2 presumably owing to depletion of nutrients or growth factors, while the bcl-2 transfectant did not until day 4 and the bcl-2 and bag-1 transfectant did not until day 5 (Figure 4b). Bcl-2 and BAG-1 co-expression delayed cell death and prolonged culture approxim-

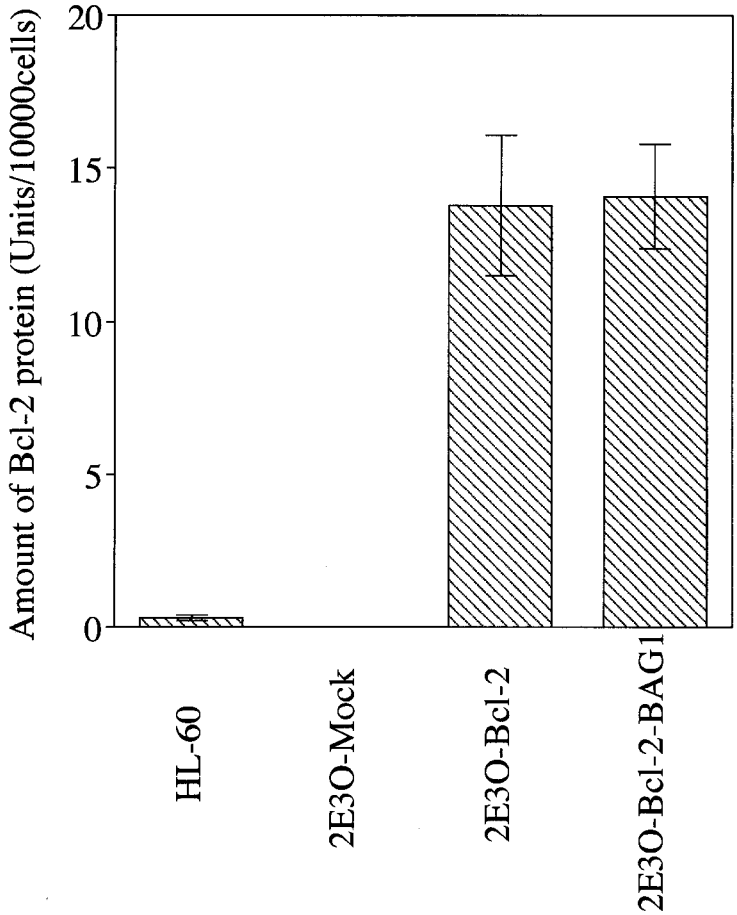

Figure 3. Measurement of Bcl-2 protein by ELISA. The hybridoma cells 2E3O-mock, 2E3O-bcl2, and 2E3O-bcl2-bag-1 were lysed and amount of Bcl-2 protein was determined by bcl-2 ELISA kit (Calbiochem, Cambridge MA). All data are normalized by the value for the population. Lysate from HL-60 human leukemia cell was included in this ELISA kit as positive control.

ately 3 days, while Bcl-2 expression prolonged culture approximately 2 days.

We have ever studied the effect of Bcl-2 expression and of Bcl-2 and BAG-1 co-expression on cell whose proliferation was arrested (Terada et al., 1997). When their proliferation was arrested by serum limitation, the bcl-2 and bag-1 co-transfectant and the bcl-2 alone transfectant survived for 3 days maintaining viability above 75% while the mock transfectant did for 1 day. BAG-1 failed to synergestically prolong the culture with Bcl-2 when the proliferation was arrested. Differently, our present results show that BAG-1 synergestically prolongs the culture with Bcl-2 when culture medium contains fetal bovine serum.

The total cell density was slightly higher in the 2E3O-Bcl2 culture than in 2E3O-Mock, probably because of cell death inhibition. After day 3, the total cell density was lower in the 2E3O-Bcl2-BAG1 culture than in 2E3O-Bcl2. As mentioned above, until day 2 the growth rate of 2E3O-Bcl2-BAG1 was as much as that of 2E3O-Bcl2. Hence, 2E3O-Bcl2-BAG1 cells stopped proliferation earlier and entered the viable non-growth phase (stationary phase) of culture, in comparison with 2E3O-Bcl2 cells.

Bcl-2 and BAG-1 cotransfection increase cell population of culture

Bcl-2 and BAG-1 co-expression delayed cell death and prolonged culture. In order to estimate the increase of production capability of culture, viable cell concentration calculated from Figure 4 was integrated with respect of culture time (Figure 5). The integrate was 1.7×10^6 cells day for 2E3O-Mock, and Bcl-2 expression increased the integrate to 2.9×10^6 cells day, and Bcl-2 and BAG-1 coexpression further increased the

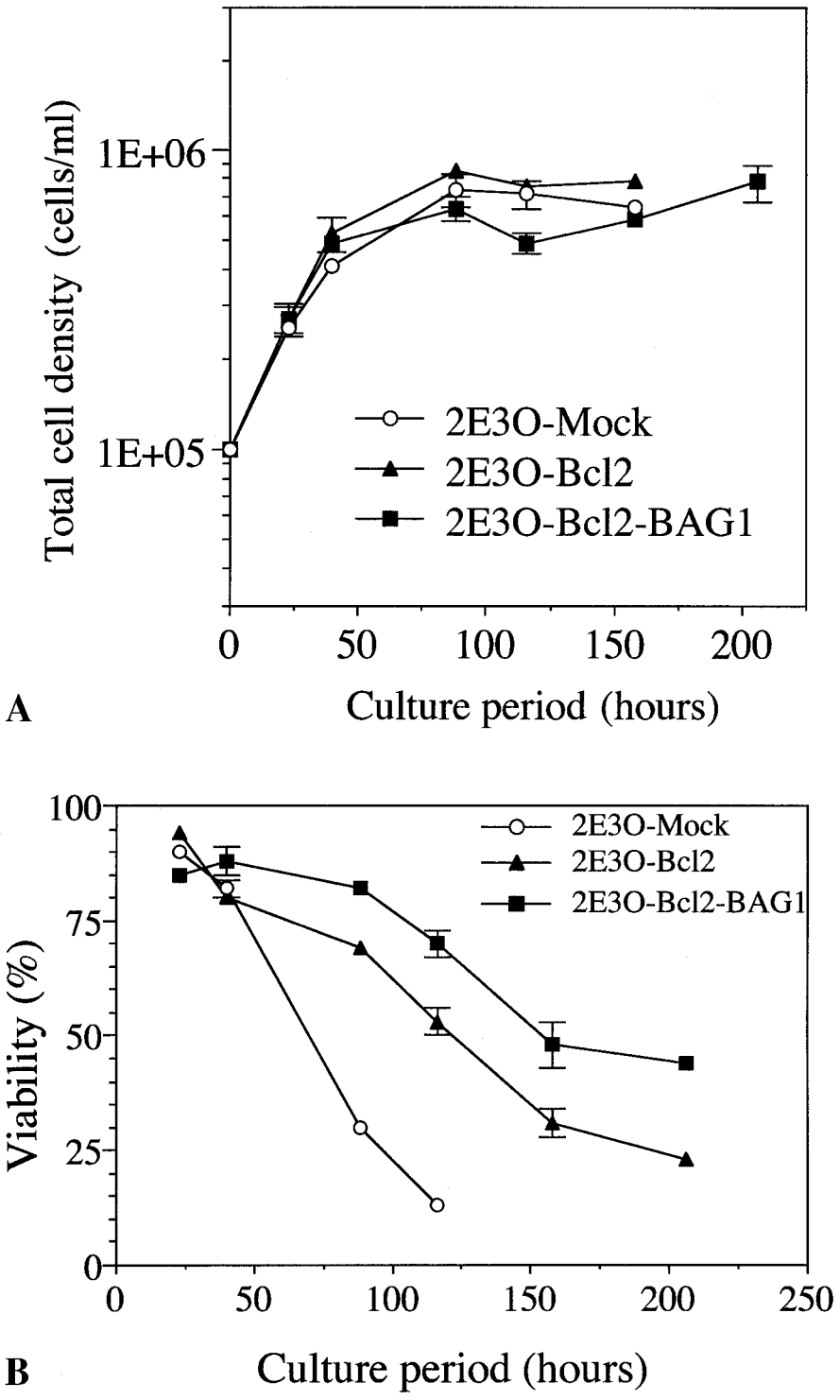

Figure 4. Growth and death of hybridoma cells expressing Bcl-2 and BAG-1. (a) Growth curves, (b) Viability. 2E3O-mock (○), 2E3O-bcl2(▲), and 2E3O-bcl2-bag-1 (■) were cultured in 24 well plates (Iwaki glass) as a batch culture.

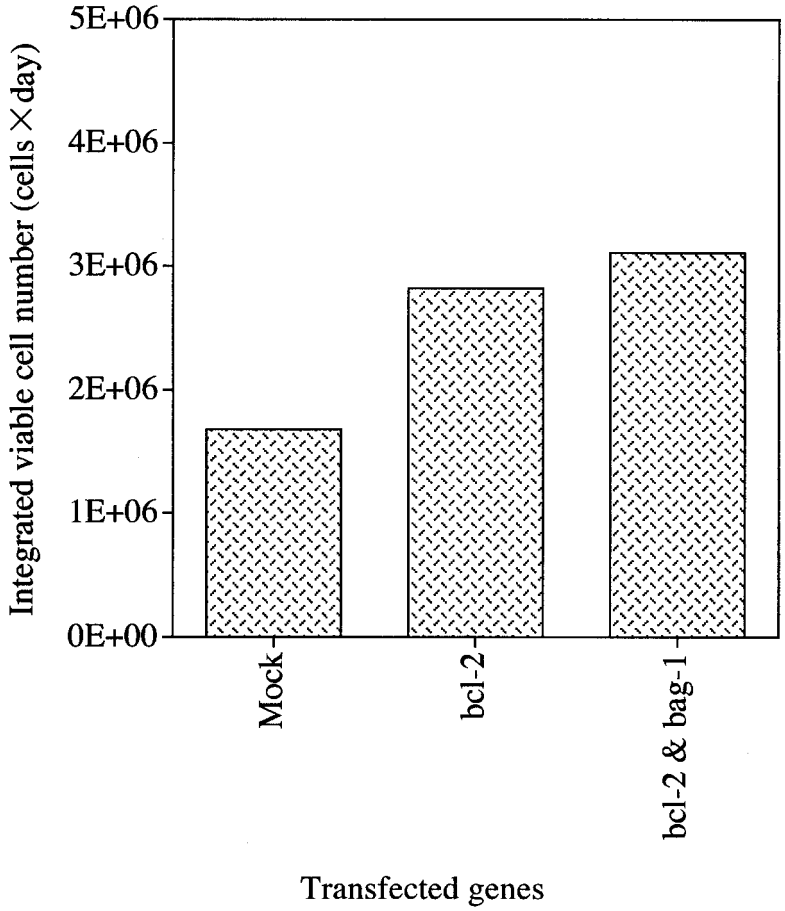

Figure 5. The integration of viable cell number of culture time. Viable cell concentrations calculated from Figure 4 were integrated of culture time with limits from culture start to termination.

integrate to 3.1×10^6 cells day. While bcl-2 expression delayed cell death 2 days and increased the cell population integrate 1.7 times as much as mock transfectant, Bcl-2 and BAG-1 co-expression delayed cell death 3 days but increased the cell population integrate only 1.8 times. Though BAG-1 synergestically prolonged culture with Bcl-2, BAG-1 slightly increased the cell population additionally.

Since we failed to establish BAG-1 solely transfectants, it could not be determined whether this increasing cell population was caused by BAG-1 alone or by Bcl-2 and BAG-1 associating effect. Further study is needed in order to determine it.

Cotransfection of bcl-2 and bag-1 enhanced MoAb production

The amount of MoAb in the cultures of which the growth and viability data are shown in Figure 4 was determined by ELISA (Figure 6). Before day 5, the Bcl-2 transfectant culture produced as much MoAb as the control culture (Figure 6a). Though the mock transfectant culture terminated at day 5, Bcl-2 transfectant continuously produced MoAb and MoAb was accumulated in the Bcl-2 transfectant culture 1.8 times more than in the mock transfectant culture (Figure 6b). As shown in Figure 5, Bcl-2 expression increased the cell population integrate 1.7 times as much as the mock transfectant. Hence, this MoAb production enhancement was due to this increase in the cell population integrate.

At day 3, Bcl-2 and BAG-1 cotransfectant culture produced more MoAb than mock or Bcl-2 transfectant culture (Figure 6a). Bcl-2 and BAG-1 cotransfectant continuously produced MoAb throughout the culture period. As a result, MoAb was accumulated in the Bcl-2 and BAG-1 co-transfectant culture 3.6 times as much as in the mock transfectant culture (Figure 6b).

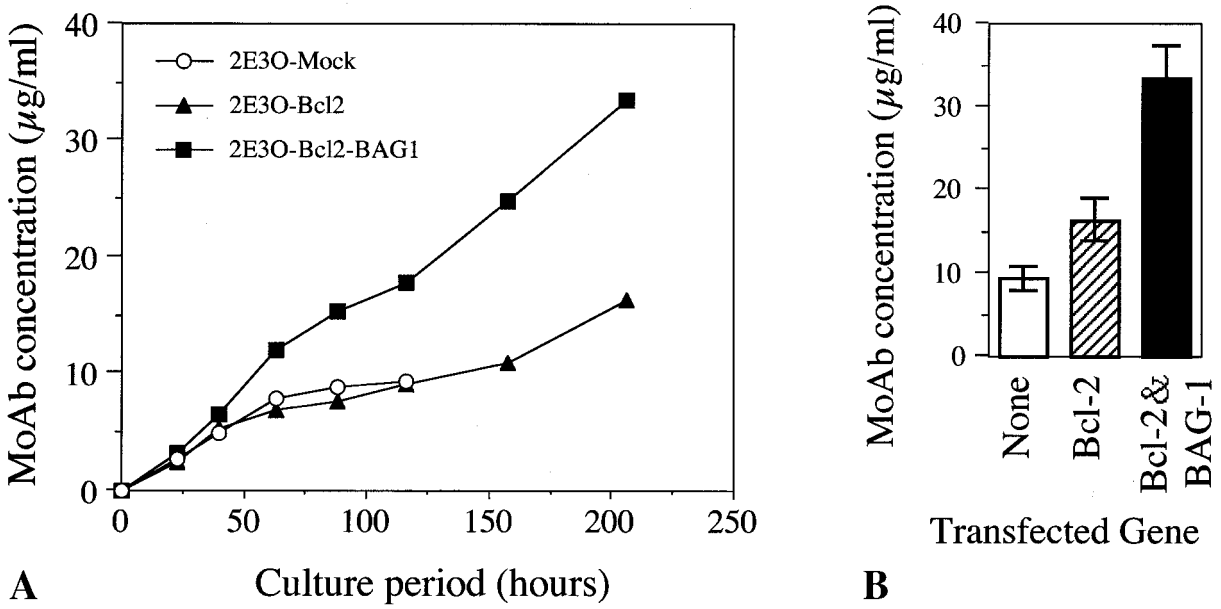

Figure 6. Bcl-2 or bcl-2 and bag-1 transfection effect on antibody production. The supernatant was taken from the culture shown in Figure 4. Antibody concentration were determined by ELISA. (a) Verses time course, (b) termination of each culture.

As shown in Figure 5, Bcl-2 and BAG-1 co-expression increased the cell population integrate only 1.8 times as much as mock transfectant. Therefore, this MoAb production enhancement was caused not only by this increase in the cell population integrate but also by enhancement of MoAb productivity per cell.

The cause of this MoAb productivity enhancement per cell is unknown. A probable explanation is: Bcl-2 and BAG-1 co-transfectant stopped proliferation earlier than mock or bcl-2 transfectants (Figure 4). This hybridoma cells produce more antibody at the slowed growth state (Makishima et al., 1992; Takahashi et al., 1994). Hence, this antiproliferation might have induced increase in MoAb production.

Another explanation is: As we reported previously, this hybridoma cells decrease MoAb production in serum starvation culture and the bcl-2 transfection failed to recover this decrease, but Bcl-2 and BAG-1 co-transfectant continuously produced MoAb in serum starvation culture (Terada et al., 1997). Therefore, Bcl-2 and BAG-1 co-transfection seemed to prevent the cells from MoAb synthesis inhibition due to serum starvation.

Conclusion

In order to increase MoAb production per culture, hybridoma cells were transfected with human bcl-2 and murine bag-1. The culture period was prolonged at least 3 days and the MoAb production per culture was enhanced fourfold for the batch culture.

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