Special Issue

Anti-apoptotic genes, *bag-1* and *bcl-2*, enabled hybridoma cells to survive under treatment for arresting cell cycle

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Abstract

Hybridoma 2E3-O cells were transfected with *bcl-2* alone or with *bcl-2* and *bag-1* in combination. The *bcl-2/bag-1* transfectant survived maintaining viability above 75% for almost 5 days when the cells were treated with excess (30 mM) thymidine for arresting cell cycle, whereas the mock transfectant survived for only 2 days, and the *bcl-2* alone transfectant lived for 4 days. Owing to this extended viable culture period, the *bcl-2/bag-1* transfectant produced twofold amount of antibody in comparison with the mock transfectant in non-proliferating state prepared by the excess thymidine treatment. When their proliferation was arrested by serum limitation, the *bcl-2/bag-1* transfectant and the *bcl-2* alone transfectant survived for 3 days maintaining viability above 75% while the mock transfectant survived only 1 day. The *bcl-2/bag-1* transfectants produced the antibody at the rate three times as high as the *bcl-2* alone transfectant and the mock transfectant in non-proliferating state established by serum limitation. Such genetic engineering of hybridoma cells for improving survival in the non-proliferating state will be useful for using nutrients in culture medium efficiently to produce antibody, since nutrients could be diverted from cell proliferation to antibody production in such non-proliferating viable cell culture.

Introduction

Method of arresting the cell cycle of mammalian cells while maintaining them viably is useful for basic research and application in the field of cytotechnology. Arresting cell cycling of hybridoma cells often enhances their antibody productivity (Suzuki *et al.*, 1992; Ramirez and Mutharasan, 1990). Antibody synthesis rate was often larger at late G1 and S phases than at the other points of the cell cycle in many hybridoma or myeloma cell lines (Al-Reubeai *et al.*, 1989; Buell *et al.*, 1969; Byars and Kidson, 1970; Garatun-Yjeldst/o *et al.*, 1971; Pryme, 1974; Takahashi *et al.*, 1969). mRNAs encoding antibodies are usually stable and hence accumulate in non-proliferating cells, resulting in increased antibody production rate (Takahashi *et al.*, 1994). In culture of non-proliferating viable hybridoma cells, resources in culture medium such as amino

acids may be diverted to synthesis of antibody from that of cell components for cell growth (Suzuki and Ollis, 1990).

Synchronous culture, which is prepared by cell cycle arrest at a specific point of the cell cycle followed by reinitiation of cell cycling, is useful for studying characteristics of each phase of the cell cycle. Culturing cells at high concentration of thymidine (Terashima and Tolmach, 1963), or under serum limitation (Zetterburg and Larson, 1985) is a technique commonly used for arresting cell cycling. Any inhibitors of DNA synthesis arrest cells; those commonly used are inhibitors of the synthesis of deoxyribonucleotide triphosphates such as thymidine at high concentrations (Johnson *et al.*, 1993). Cells located outside S phase at the time of excess thymidine addition are arrested when they pass through the G1/S boundary. For preparing synchronous culture, a double thymidine treatment, in which cells

are arrested anywhere in S by giving excess thymidine, released by eliminating thymidine, and arrested again by giving excess thymidine when the population has moved out of S, is useful in order to accumulate cells close to the G1/S boundary (Stein and Borun, 1972). The cells accumulated at G1/S boundary are released from arrest by culturing at reduced thymidine concentration, and then synchronously progress in the cell cycle. However, prolonged or double treatment with excess thymidine can be toxic to some cell lines. For example, hybridoma cells are not sturdy enough to maintain high viability during thymidine treatment. Hence, the excess thymidine method can not be applied for establishing arrested or synchronous culture of hybridoma cells.

Serum deprivation arrests cell cycle firstly at early G1 and then the arrested cells enter in G0 (Zetterburg and Larson, 1985). However, it induces apoptosis, suicidal cell death, of many kinds of cells including hybridoma cells (Singh *et al.*, 1994; Mercille and Massie, 1994). No practical methods of establishing viably arrested culture of hybridoma cells have been developed. Our experience of applying various methods of growth-suppression to hybridoma cells suggested that suppression of hybridoma proliferation usually induces apoptosis. Therefore, in this work, we tried to engineer hybridoma cells by using apoptosis suppressing genes, *bcl-2* and *bag-1*, to make them resistant to apoptosis and durable in the excess thymidine treatment and the low serum culture.

bcl-2 is a gene known to suppress multiple forms of apoptosis (Patterson *et al.*, 1992; Reed 1994). High level expression of human *bcl-2* protein resulted in the better survival of mouse myeloid cells in the absence of the growth factor (Tsujimoto, 1989) and lymphokine (Vaux *et al.*, 1988). Deregulated *bcl-2* expression prolonged survival of growth factor-deprived hemopoietic cell lines (Nunez *et al.*, 1990). The high levels of the *bcl-2* gene product protected human B cells from a variety of agents giving stress including heat shock, ethanol, DNA-damaging agents, and serum deprivation (Tsujimoto, 1989; Miyashita and Reed 1992).

Recently, BAG-1, Bcl-2 association athagogene 1, protein was discovered (Takayama *et al.*, 1995). Co-expression of *bag-1* and *bcl-2* can provide markedly increased protection from cell death induced by several stimuli. In addition, BAG-1 binds to Hsp 70/Hsc70 family proteins, modulating their activities, and providing protection against apoptosis and cellular stress (Takayama *et al.*, 1997).

Cell engineering using these apoptosis suppressing genes enabled hybridoma cells to survive longer in non-proliferating state induced by excess thymidine treatment or by serum limitation.

Materials and methods

Cell line and culture condition

The 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% vol/vol FBS (JRH Bioscience, lot. 4C2042), 20 mM HEPES, 0.2% NaHCO₃, 2 mM glutamine, and 0.06 mg ml⁻¹ kanamycin. The cells were grown in 24 well plate (Iwaki glass) at 37 °C in humidified air containing CO₂ at 5%.

Transfection

The vector BCMGneo-bcl-2 for expressing human *bcl-2* (Tsujimoto, 1989) in the hybridoma cells was prepared as reported elsewhere (Itoh *et al.*, 1995). The expression vector pZeo-bag-1 was prepared by inserting murine *bag-1* cDNA (Takayama *et al.*, 1995) into pZeoSV vector (Invitrogen). Hybridoma 2E3-O was transfected with BCMGneo vector (Karasuyama *et al.*, 1990), with BCMGneo-bcl-2 and pZeo vectors, and with BCMGneo-bcl-2 and pZeo-bag-1 vectors, respectively, and named mock transfectant 2E3O-mock, *bcl-2* transfectant 2E3O-bcl-2, and *bcl-2/bag-1* transfectant 2E3O-bcl-2/bag-1. The transfection method was electroporation. For selecting successfully transfected cells, 2E3O-mock was cultured in the presence of H418 (Gibco) at 0.25 mg ml⁻¹, and 2E3O-bcl-2 and 2E3O-bcl-2/bag-1 were cultured in the presence of 0.25 mg ml⁻¹ G418 (Gibco) and 0.25 mg ml⁻¹ Zeocin (Invitrogen). Viable and dead cells were determined by counting in a hemocytometer under a phase contrast microscope using trypan blue exclusion. All experiments were performed in duplicate or triplicate.

Figure 1A.

Figure 1C.

Figure 1B.

Figure 1A–D. Growth and death of *bcl-2* or *bcl-2/bag-1* transfected hybridoma cells under excess thymidine treatment. The mock transfectant, *bcl-2* transfectant, and *bcl-2/bag-1* cotransfectant were prepared as described in Materials and methods. The three transfectants were cultured at various concentrations of thymidine for 8 days. A, B, and C: growth curves of the mock (A), *bcl-2* (B), and *bcl-2/bag-1* (C) transfectants cultured with thymidine at 0 (□), 10 (▲) or 30 (●) mM thymidine. D: viability of mock transfectant (□), *bcl-2* transfectant (▲), *bcl-2/bag-1* cotransfectant (●) cultured with 30 mM thymidine.

Cell cycle arrest by excess thymidine

Exponentially growing cells are harvested from cultures by centrifugation, and resuspended in the standard culture medium supplemented with thymidine (Wako Junyaku, Osaka) at various concentrations.

Cell cycle arrest by serum limitation

Exponentially growing cells were harvested from cultures by centrifugation, washed twice with DME medium, and resuspended in the standard culture medium

supplemented with FBS at a concentration of 0.2% instead of 10%.

Determination of antibody concentration

The antibody concentration was determined by ELISA (Engvall, 1980). The antibody 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, Inc.-Tago Products). Amount of the antibody was determined by measuring absorbance, using o-phenylenediamine dihydrochloride as substrate for the peroxidase. A known amount of the purified antibody secreted by 2E3-O cells was used as standard.

Results and discussion

Bcl-2 and *bcl-2/bag-1* transfectants survived longer when arrested with excess thymidine

Hybridoma 2E3-O cells expressing *bcl-2* alone (2E3/*bcl-2*), those coexpressing *bcl-2* and *bag-1* (2E3O-*bcl-2/bag-1*), and those transfected with vector BCMGS-neo alone (mock transfectant 2E3O-mock) were prepared as described in Materials and Methods. The expression of *bcl-2* and *bag-1* proteins was confirmed by western blot using anti-human Bcl-2 antibody and anti-mouse BAG-1 antibody, respectively (data not shown). These transfectants were cultured with 0, 10 or 30 mM thymidine in the culture medium described in Methods and Materials. Figure 1-A, B, and C show the growth curves of the mock, the *bcl-2* alone, and the *bcl-2/bag-1* double transfectants, respectively. Proliferation of all the three transfectants was similarly suppressed by thymidine in dose dependent manner, being completely suppressed at 30 mM of thymidine. The cell cycle turned round two to three times initially, before thymidine at 10 mM completely suppressed proliferation of all the three transfectants.

Figure 1-D shows the viability of the three transfectants cultured with thymidine at 30 mM. The *bcl-2* transfection improved survival of the non-proliferating hybridoma cells under the excess thymidine treatment, prolonging the viable culture period maintaining viability above 75% from 2 days to 4 days while the mock transfectant survived for 2 days. Coexpression of *bag-1* with *bcl-2* further improved survival of the non-proliferating cells. Such improvement of survival of

Figure 2A.

Figure 2A-B. Growth and death of *bcl-2* or *bcl-2/bag-1* transfected hybridoma cells under serum limitation. The mock transfectant ($\square$), *bcl-2* transfectant ($\blacktriangle$), and *bcl-2/bag-1* cotransfectant ($\bullet$) prepared as described in Materials and methods were cultured at low (0.2%) serum concentration for 7 days. A: growth curves, B: viability.

the hybridoma cells during excess thymidine treatment will make the preparation of synchronous culture using the double thymidine treatment more successful.

Bcl-2 and *bcl-2/bag-1* transfectants survived longer when arrested by serum limitation

The *bcl-2* transfectant, the *bcl-2/bag-1* cotransfectant, and the mock transfectant were cultured at various concentrations of fetal bovine serum (FBS). All three transfectants ceased proliferating when the FBS concentration was reduced to 0.2% (vol/vol). Figure 2-A and -B show the growth curves and the viability, respective-

Figure 3. Antibody production of *bcl-2* or *bcl-2/bag-1* transfected hybridoma cells in non-proliferating state prepared by excess thymidine treatment. The mock, *bcl-2*, and *bcl-2/bag-1* transfectants were cultured with 30 mM thymidine for 8 days. The corresponding growth and viability data are shown in Figure 1. The antibody concentrations in the supernatants sampled from the cultures were determined by ELISA for mock transfectant (□), *bcl-2* transfectant (▲), and *bcl-2/bag-1* transfectant (●).

ly, of the three transfectants cultured with 0.2% FBS. The *bcl-2* transfection improved survival of the non-proliferating hybridoma cells under serum limitation, prolonging the viable culture period maintaining viability above 75% from 1 day to 4 days. Cotransfection of *bag-1* which *bcl-2* did not further improve the survival in comparison with the *bcl-2* alone transfection.

Bcl-2 and bcl-2/bag-1 transfectants produced more antibody when arrested with excess thymidine

Antibody concentrations in the culture supernatant of the three transfectants cultured with excess thymidine at 30 mM are shown in Figure 3. The *bcl-2/bag-1* cotransfectant produced twofold more antibody in comparison with the mock transfectants. This increased production was due to the prolonged viable culture period, but not to the increased antibody production rate of single cell as discussed below.

Figure 4 shows the specific antibody production rate, antibody production rate per viable cell, of the three transfectants cultured with thymidine at 0, 10, and 30 mM. The rate was calculated as the amount of the antibody produced between 66 h and 137 h of the cultures divided by the viable cell number integrated with respect to time in the interval. The specific production rate of the mock transfectant increased and the

Figure 4. Specific antibody production rate of *bcl-2* or *bcl-2/bag-1* transfected hybridoma cells in proliferating (no thymidine) or non-proliferating (excess thymidine) state. The specific antibody production rates, antibody production per cell and day, were calculated between 66 or 97 h and 137 h of the cultures of which the growth data are shown in Figure 1, and are represented by open bars for no thymidine, hatched bars for 10 mM thymidine, and solid bars for 30 mM thymidine.

growth rate decreased as thymidine concentration was raised. This negative correlation between the antibody production rate and the growth rate is consistent with the reported data (Suzuki and Ollis, 1990; Takahashi *et al.*, 1994). However, the specific antibody production rate of the *bcl-2* transfectant and the *bcl-2/bag-1* cotransfectant did not increase with decreasing growth rate. In other words, both the exponentially growing and the non-growing *bcl-2* or *bcl-2/bag-1* transfectants produced antibody at the similar rates in these cases. This different correlation between the specific antibody production rate and the growth rate can be a clue for studying anti-apoptotic mechanism involving *bcl-2* and *bag-1* proteins. For example, it may suggest that under the excess thymidine condition *bcl-2* protein suppresses synthesis of mRNAs which are unnecessary for survival.

Bcl-2/bag-1 cotransfectant produced antibody at threefold rate when arrested with serum limitation

Figure 5 shows the specific antibody production rate of the three transfectants completely arrested as shown in Figure 4 by culturing in the medium supplemented with ABS at 0.2% instead of standard 10%. While the three transfectants and the untransfected 2E3-/O (data not shown) produced the antibody at the rate of 10 pg cell⁻¹ d⁻¹ in proliferating state (data at 0 mM thymi-

dine in Figure 4), all the three transfectants did at the increased rate of 20 to 30 pg cell⁻¹ d⁻¹ in the non-proliferating state. This increase in antibody production rate associated with suppression of proliferation is consistent with the reported data (Suzuki and Ollis, 1990; Takahashi *et al.*, 1994) and with the data for the mock transfectant arrested by 10 or 30 mM thymidine shown in Figure 4. It is noticeable that among the three transfectants, the *bcl-2/bag-1* cotransfectant produced the antibody at the highest rate and maintained the high rate through the culture, while the other transfectants lost their enhanced productivity rapidly. The reason of this synergetic effect of *bcl-2/bag-1* cotransfection is unknown because how *bcl-2* blocks apoptosis is not elucidated. But recently two protein that bind to BAG-1 were reported (Bardelli *et al.*, 1996; Wang *et al.*, 1996). One is Raf-1 and another is hepatocyte growth factor receptor. Raf-1 is major member in nitogen-activated protein kinase (MARK) cascade. It is well-known that the MARK cascade plays a crucial role in the transduction of extracellular signals into responses governing growth and differentiation. Thus BAG-1 associates with a certain regulating factor such as Raf-1 and might regulate protein synthesis or cell differentiation.

The culture of the *bcl-2/bag-1* cotransfectant produced the antibody fourfold in total owing to the longer maintained high specific production rate and high viability in comparison with the mock transfectants.

Conclusion

We genetically modified the hybridoma 2E3-O cells by transfecting with anti-apoptotic genes *bcl-2* and *bag-1* so that they can survive in non-proliferating state prepared by excess thymidine treatment or by serum limitation. Such genetic engineering of hybridoma cells will be applied for keeping the cells viable during double thymidine treatment to establish synchronous culture for cell cycle analysis.

Using these apoptosis-resistant hybridoma cells, non-proliferating viable hybridoma cell culture can be established by applying excess thymidine treatment or serum limitation. In such non-proliferating viable cell culture, the hybridoma cells can utilize nutrients in medium efficiently for antibody production by diverting them from cell growth to antibody production.

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