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Response of recombinant Chinese hamster ovary cells to hyperosmotic pressure: effect of Bcl-2 overexpression

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Abstract

In an attempt to use the hyperosmotic pressure for improved foreign protein production in recombinant Chinese hamster ovary (rCHO) cells, the response of rCHO cells producing a humanized antibody (SH2-0.32-Δbcl-2 cells) to hyperosmotic pressure was determined in regard to cell growth and death, and antibody production. Further, the feasibility of Bcl-2 overexpression in improving rCHO cell viability under hyperosmotic pressure was also determined by comparing control cells (SH2-0.32-Δbcl-2) with Bcl-2 overexpressing cells (14C6-bcl-2). After 3 days of cultivation in the standard medium (294 mOsm kg^{-1}), the spent medium was exchanged with the fresh media with various osmolalities (294–640 mOsm kg^{-1}). The results obtained show that hyperosmotic pressure inhibited cell growth in a dose-dependent manner, though 14C6-bcl-2 cells were less susceptible to hyperosmotic pressure than SH2-0.32-Δbcl-2 cells. At 522 mOsm kg^{-1}, SH2-0.32-Δbcl-2 cells underwent a gradual cell death mainly through apoptosis due to the cytotoxic effect of hyperosmotic pressure. In contrast, Bcl-2 overexpression in 14C6-bcl-2 cells could delay the apoptosis induced by 522 mOsm kg^{-1} by inhibiting caspase-3 activation. Bcl-2 overexpression could also improve the cellular membrane integrity of 14C6-bcl-2 cells. When subjected to hyperosmotic pressure, the specific antibody productivity of SH2-0.32-Δbcl-2 cells and 14C6-bcl-2 cells was increased in a similar extent. As a result, the final antibody concentration achieved in 14C6-bcl-2 cells at 522 mOsm kg^{-1} was 2.5-fold higher than that at 294 mOsm kg^{-1}. At 580 mOsm kg^{-1}, acute hyperosmotic pressure induced the rapid loss of viability in both SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells through necrosis rather than through apoptosis. Taken together, Bcl-2 overexpression and optimized hyperosmotic pressure could improve the antibody production of rCHO cells. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Antibody; Apoptosis; Bcl-2 overexpression; Chinese hamster ovary (CHO) cells; Hyperosmotic pressure

1. Introduction

A high foreign protein productivity in recombinant Chinese hamster ovary (rCHO) cell cultures, which is desired for economical production, can be achieved by increasing cell concentration and/or specific foreign protein productivity (q).

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Among various methods to increase *q*, hyperosmotic pressure, which can be induced by addition of cheap salts to culture media, has been recognized as being an economical solution to increase *q* in hybridoma (Cherlet and Marc, 1999; Oh et al., 1995; Ozturk and Palsson, 1991; Ryu and Lee, 1996), transfectoma (Lee and Lee, 2000), and rCHO cell cultures (Kim et al., 2000; Ryu et al., 2000). Without intentional addition of salts to culture media, hyperosmolar culture condition can also occur naturally in fed-batch culture or high cell density culture. In fed-batch culture, repeated feeding of medium concentrates leads to substantial increase in medium osmolality (Bibila and Robinson, 1995; Ryu and Lee, 1999). In high cell density culture, hyperosmolality is induced due to high HCO_3^- concentration caused by elevated CO_2 partial pressure as well as increased cation concentration resulting from the addition of base for pH control (Schmelzer et al., 2000).

Despite the potential of commercial strategies based on hyperosmotic pressure, the use of hyperosmolar culture medium has not been popular because cell growth is depressed at elevated osmolality. Hence, the enhanced *q* in hyperosmolar batch culture does not result in a substantial increase in the final product concentration (Cherlet and Marc, 1999; Lee and Park, 1995; Ozturk and Palsson, 1991). Depressed cell growth at elevated osmolality is probably because hyperosmotic pressure induces the cellular apoptotic cell death, as observed in various cell types, like rat alveolar type II cells (Edwards et al., 1998), rat cardiac fibroblast cells (Mockridge et al., 2000), human neuroblastoma cells (Singleton et al., 1996; Van Golen and Feldman, 2000), and human umbilical vein endothelial cells (HUVEC, Wu et al., 1999).

The apoptotic cell death induced in mild suboptimal conditions is an active, genetically controlled process of cell suicide mediated by the activation of a series of caspases. Therefore, apoptotic cell death can be regulated to some extent by genetic modification like the expression of a family of Bcl-2 survival proteins (Kim and Lee, 2001; Perani et al., 1998; Simpson et al., 1997). The overexpression of *bcl-2* enabled adaptation of hybridoma cells to 400 mOsm kg^{-1} medium, which was not possible in the control cell line (Perani et al., 1998). In contrast, necrotic cell death induced at high stress levels is a passive, genetically uncontrolled death. Thus, cells are likely to follow different death modes, apoptosis or necrosis, depending on the degree of osmotic stress. In fact, human endothelial cells which showed apoptotic cell death in the medium with 30 mM glucose lost their viability by necrotic cell death in the medium with 60 mM glucose (Wu et al., 1999). For the success of hyperosmolar rCHO cell culture, cell death modes under hyperosmolality need to be verified. Cell death may also affect the recovery efficiency and the integrity of protein product by liberating to the culture supernatant intracellular enzymes, like proteases and glycosidases that may degrade the product, as well as intracellular proteins that will contaminate the product and render downstream process more difficult. However, to our knowledge, there are no systematic studies on the effect of hyperosmotic pressure on the cell death modes of rCHO cells.

In this study, we investigate the effect of hyperosmotic pressure on rCHO cells expressing humanized antibody in regard to cell growth and death, and antibody production. Furthermore, we also investigate whether the hyperosmolality-induced apoptosis can be suppressed by overexpression of *bcl-2* in rCHO cells and thereby, can result in an increased production of humanized antibody.

2. Materials and methods

2.1. Cell lines

The rCHO cell line producing a humanized antibody against the S surface antigen of hepatitis B virus (SH2-0.32) was established in our laboratory. The cell lines used in this study were 14C6-bcl-2 with *bcl-2* and SH2-0.32-Δbcl-2 without *bcl-2*. They were constructed by transfecting SH2-0.32 cells with the pBcl-2/Zeo and the null pcDNA3.1/Zeo(+) without *bcl-2*, respectively (Kim and Lee, 2001).

2.2. Culture maintenance, preparation of hyperosmolar culture medium, and cell culture

The medium for culture maintenance was α-minimal essential medium (α-MEM, Gibco, Grand Island, NY) supplemented with 5% dialyzed fetal bovine serum (dFBS, Gibco) and 0.32 μM methotrexate (MTX, Sigma, St. Louis, MO). The cells were maintained as monolayer cultures in 25-cm^2 T-flasks (Nunc, Roskilde, Denmark) in a 5% CO_2/air mixture, humidified at 37 °C.

Hyperosmolar culture media with various osmolalities were prepared by adding NaCl to the standard medium with physiological osmolality (α-MEM supplemented with 5% dFBS and 0.32 μM MTX). The osmolality of the standard medium was 294 mOsm kg^{-1} and of the hyperosmolar culture media was 459, 522, 580, and 640 mOsm kg^{-1}. No antibiotics were added to the culture medium.

Identical batch cultures were carried out with the two rCHO cell lines. Cells exponentially growing in the standard medium (physiological osmolality) were inoculated into 6-well tissue culture plates (Nunc) containing 5 ml of α-MEM supplemented with 5% dFBS and 0.32 μM MTX. Initial cell density was 4×10^4 cells ml^{-1}. After 3-day cultivation, the spent medium was replaced with 5 ml of fresh culture media with various osmolalities (294–640 mOsm kg^{-1}). Periodically, cells were detached from the culture plate by trypsinization, and cell concentration and viability were determined. Culture supernatants were aliquoted and kept frozen at −70 °C for further study. The cultures were performed in a 5% CO_2/air mixture, humidified at 37 °C.

2.3. Cell concentration, viability, and antibody assays

Cell concentration was estimated using a hemacytometer. Viable cells were distinguished from dead cells using trypan blue dye exclusion method. Secreted humanized antibody concentration was measured by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (Kim et al., 1996). In brief, 96-well microtiter plates were coated with goat *anti*-human IgG (whole molecule, Sigma) and blocked with bovine serum albumin (BSA) and Tween 20. Purified humanized antibody was used as a standard, and horseradish peroxidase (HRP)-conjugated goat *anti*-human IgG (Fc specific, Sigma) was used as an enzyme–antibody conjugate.

2.4. Determination of lactate dehydrogenase activity in culture supernatant

After thawing the aliquoted, frozen culture supernatant, lactate dehydrogenase (LDH) activity in culture supernatant was determined using a commercial test kit (Sigma, Catalog No. 228-10). In order to avoid the day-to-day variations of LDH activity assay, the supernatant of cultured medium was kept frozen at −70 °C until the LDH activity was measured. After one cycle of freezing and thawing, more than 80% of the LDH activity of sample was retained. The detection principle was based on the fact that LDH catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of NAD to NADH, and the formation of NADH results in an increase in absorbance at 340 nm. The rate of increase in OD at 340 nm is directly proportional to LDH activity in culture supernatant. One unit (U) of LDH activity is defined as the amount of enzyme that will catalyze the formation of 1 μmol of NADH per minute.

2.5. Quantitation of apoptosis and necrosis

The cells undergoing apoptosis were discriminated from the cells undergoing necrosis by staining cells with DNA binding fluorescent dyes, acridine orange (AO, Molecular Probes, Eugene, OR) and ethidium bromide (EB, Sigma). The procedure used was based on the protocols as described previously (Mercille and Massie, 1994; Zanghi et al., 1999). Cells were suspended at 5×10^5–5×10^6 cells ml^{-1} in Ca^{+2} and Mg^{+2}-free phosphate buffered saline (PBS, Gibco) and stained with 1.5 μg ml^{-1} AO and 7.5 μg ml^{-1} EB. The cell mixture was examined under a Nikon Microphot-FXA epifluorescence microscope. Viable and dead cells were distinguished by bright-green and -orange nuclei, respectively.

Apoptotic cells were distinguished from necrotic cells by the observation of a fragmented or of a non-fragmented nucleus, respectively. A minimum of 200 total cells were counted and identified as one of four groups according to their cellular states; viable with non-fragmented nucleus (VNA; viable, non-apoptotic); viable with fragmented nucleus (VA; viable, apoptotic); non-viable with fragmented nucleus (NVA; non-viable, apoptotic); non-viable with non-fragmented nucleus (NVN; non-viable, necrotic).

2.6. Measurement of caspase-3 activity

Cells were seeded at a concentration of 1×10^5 cells ml^{-1} in 100 mm culture dishes (Nunc) containing 10 ml of α-MEM supplemented with 5% dFBS and 0.32 μM MTX. After 3-day cultivation, the spent medium was replaced with 10 ml of fresh hyperosmolar medium. Thereafter, total cells (viable and non-viable) were harvested from culture dishes at regular time intervals. Cell lysates were prepared using lysis buffer (1% NP-40, 0.1% SDS, 0.02% NaN_3, 50 mM Tris (pH 8.0), 150 mM NaCl, 100 μg ml^{-1} phenylmethanesulfonyl fluoride (PMSF), and 1 μg ml^{-1} aprotinin) as described previously (Kim and Lee, 2001). Caspase-3 activities in cell lysates were measured by observing cleavage of caspase-3 substrate, poly (ADP-ribose) polymerase (PARP) in Western blot as described previously (McKenna and Cotter, 2000). PARP cleavage was determined by a Western blot analysis of cell lysate using an *anti*-PARP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as a primary antibody and a HRP-conjugated *anti*-rabbit IgG goat polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) as a secondary antibody. Intact and cleaved PARPs were detected using an ECL Western blotting system (Amersham Pharmacia Biotech, Piscataway, NJ).

3. Results

3.1. Effect of hyperosmotic pressure on cell growth and antibody production

To determine the effect of hyperosmotic pressure on cell growth and antibody production, control (SH2-0.32-Δbcl-2) and Bcl-2 overexpressing cells (14C6-bcl-2) were first cultivated in the standard medium (294 mOsm kg^{-1}). After 3 days of cultivation, the spent medium was exchanged with the fresh media with various osmolalities (294–640 mOsm kg^{-1}).

Fig. 1 shows the characteristics of cell growth, viability, and antibody production. Cell growth of both SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells was depressed at higher osmolalities, though 14C6-bcl-2 cells were more resistant to hyperosmotic pressure than SH2-0.32-Δbcl-2 cells.

After medium exchange to the fresh standard medium (294 mOsm kg^{-1}), both SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells continued to grow until the maximal viable cell concentrations reached approximately 5.0×10^5 and 8.0×10^5 ml^{-1}, respectively. Thereafter, the viable cell concentration of SH2-0.32-Δbcl-2 cells began to decrease rapidly and the cell viability fell down to approximately 50% at the end of culture. In contrast, the viable cell concentration of 14C6-bcl-2 cells remained at about 7.0–8.0×10^5 ml^{-1} without any considerable loss of viability throughout the cultivation period (viability $> 80\%$), though glucose was depleted completely 2 days after medium exchange (data not shown).

After medium exchange to the hyperosmolar culture medium, the cytotoxic effect of hyperosmotic pressure became apparent above 459 mOsm kg^{-1} medium. In 522 mOsm kg^{-1} medium, SH2-0.32-Δbcl-2 cells did not show any observable cell growth while 14C6-bcl-2 cells showed a little cell growth after 1.5-day adaptation period and maintained relatively high cell viability throughout the cultivation period ($> 70\%$). Despite the depressed cell growth, the final antibody concentrations of SH2-0.32-Δbcl-2 and 14C6-bcl-2 cell cultures in 522 mOsm kg^{-1} medium were enhanced by 2.1- and 2.5-fold, respectively, as compared with those obtained in the standard medium. The higher final antibody concentration in 522 mOsm kg^{-1} medium was achieved probably because the beneficial effect of hyperosmotic pressure on specific antibody productivity (q_{Ab}) outweighed its detrimental effect on cell growth.

In 580 mOsm kg^{-1} medium, viabilities of SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells decreased rapidly to 50% within 24 h. Despite the significant cell death, the final antibody concentration was similar to that in the standard medium, indicating that q_{Ab} was significantly elevated at 580 mOsm kg^{-1}. In 640 mOsm kg^{-1}, SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells could not survive. Thus, we further investigated the effect of hyperosmotic pressure on other cellular responses in the range of 294–580 mOsm kg^{-1}.

Fig. 2 shows the time integral of viable cells versus antibody concentration plots used to determine q_{Ab} for cultures shown in Fig. 1. The slope of such a plot is equal to q_{Ab} if q_{Ab} is constant. As shown in Fig. 2A and B, q_{Ab} was not constant throughout the culture. Two phases can be distinguished in terms of q_{Ab} value before and after the specific point (as indicated by arrows); antibody production phases with a constant q_{Ab} (left sides of the arrows) and lower or no antibody production phases (right sides of the arrows). Until reaching the specific culture time indicated by the arrows (antibody production phase), q_{Ab} was constant. Thereafter, q_{Ab} was significantly decreased probably because of either the nutrient limitation at 294 and 459 mOsm kg^{-1} or cell death at 522 and 580 mOsm kg^{-1}. Because most of antibodies were produced during antibody production phase, q_{Ab} was calculated from the slope at this phase and plotted as a function of osmolalities (Fig. 2C). The q_{Ab} of SH2-0.32-Δbcl-2 and 14C6-bcl-2

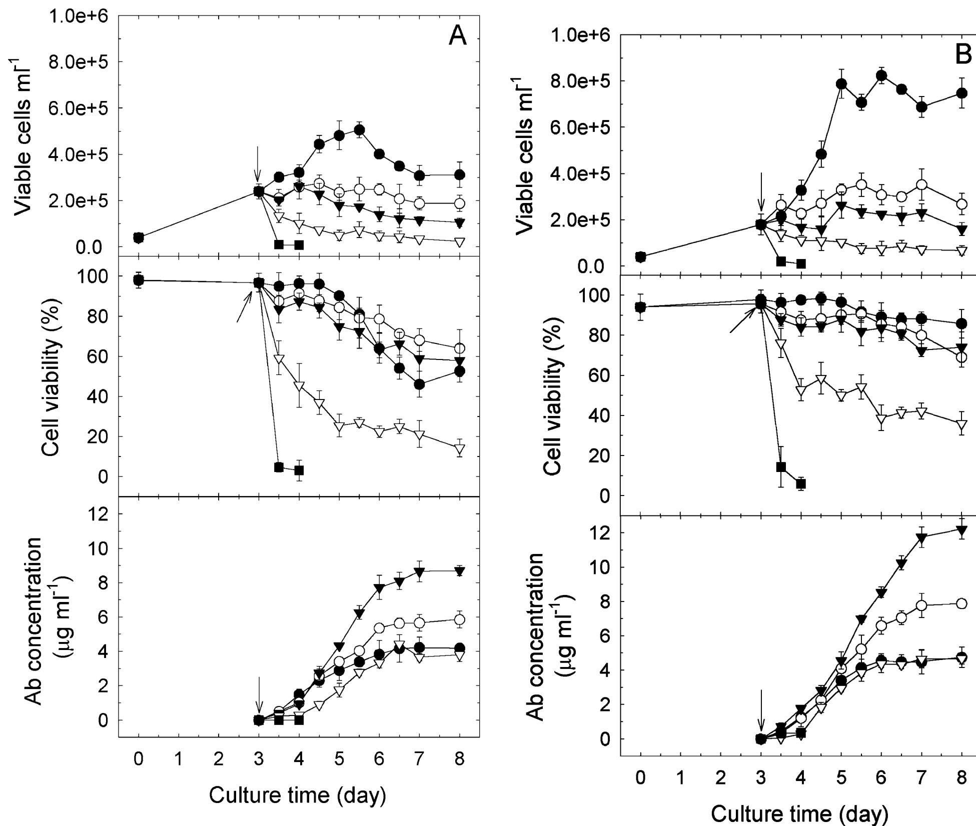

Fig. 1. Cell growth, cell viability, and antibody concentration in: (A) SH2-0.32-Δbcl-2; and (B) 14C6-bcl-2 cell cultures at various osmolalities. ●, 294 mOsm kg^{-1}; ○, 459 mOsm kg^{-1}; ▼, 522 mOsm kg^{-1}; ▽, 580 mOsm kg^{-1}; ■, 640 mOsm kg^{-1}. The arrows indicate the time when the spent medium was exchanged with the fresh media with various osmolalities. Error bars represent the standard deviations calculated from data obtained in duplicate experiments.

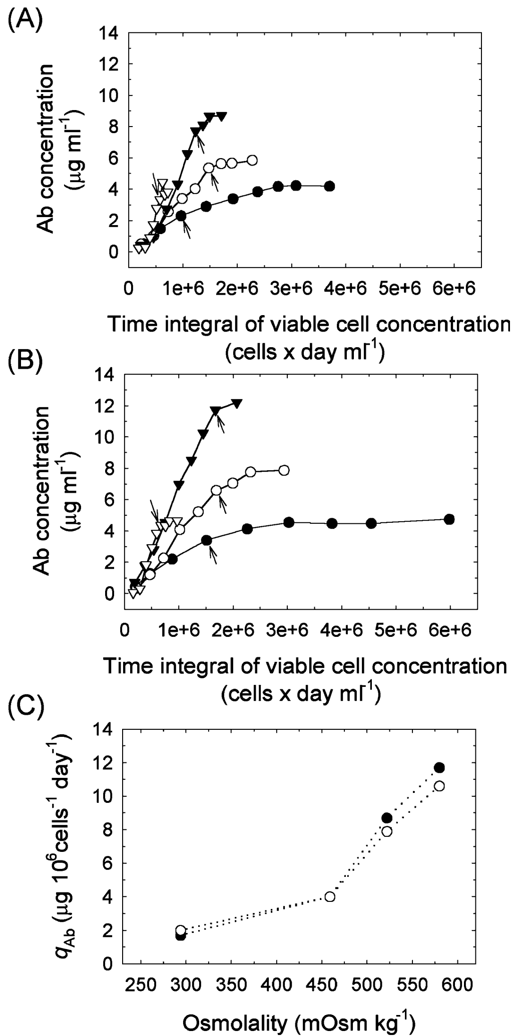

Fig. 2. Effect of hyperosmotic pressure on antibody production. Time integral of viable cells versus antibody concentration in: (A) SH2-0.32-Δbcl-2; and (B) 14C6-bcl-2 cell cultures. ●, 294 mOsm kg^{-1}; ○, 459 mOsm kg^{-1}; ▼, 522 mOsm kg^{-1}; ▽, 580 mOsm kg^{-1}. (C) The cell specific antibody productivity (q_{Ab}) at various osmolalities in SH2-0.32-Δbcl-2 (●) and 14C6-bcl-2 (○) cell cultures. Time integral of viable cells versus antibody concentration was plotted for q_{Ab} evaluation. Two phases were distinguished; antibody production phases with a constant q_{Ab} (left sides of the arrows) and lower or no antibody production phases (right sides of the arrows). The q_{Ab} was calculated from the slopes of antibody production phases.

cells was increased to a similar extent at hyperosmolalities, indicating that overexpression of Bcl-2 does not influence the response of rCHO cells to hyperosmotic pressure in regard to q_{Ab}. The q_{Ab} of SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells at 580 mOsm kg^{-1} was 11.7 and 10.6 μg per 10^6 cells per day, respectively. Thus, the final antibody concentration of 14C6-bcl-2 cells was higher than that of SH2-0.32-Δbcl-2 cells at hyperosmolalities because Bcl-2 overexpression made 14C6-bcl-2 cells more resistant to hyperosmotic pressure, resulting in increased time integral of viable cells.

3.2. Determination of cellular membrane disintegration under hyperosmotic pressure

Cell viability determined by trypan blue dye exclusion method does not account for cell lysis. The cell lysis following cell death can influence the quality of protein product because various proteases and glycosidases liberated from non-viable cells with ruptured membrane influence the molecular integrity of protein products (Goldman et al., 1997; Hansen et al., 1997; Teige et al., 1994). The amount of lysed cell can be measured by measuring the amount of LDH released into the culture supernatant (Cruz et al., 2000). Thus, to determine the degree of cell lysis, the LDH activity in culture supernatant was measured daily after the introduction of hyperosmotic pressure. The LDH activity was not affected by the presence of NaCl in the hyperosmolar medium used in this study (data not shown).

Fig. 3 shows the profiles of LDH activity after medium exchange during SH2-0.32-Δbcl-2 and 14C6-bcl-2 cell cultures shown in Fig. 1. The LDH activity of SH2-0.32-Δbcl-2 cells began to increase significantly 2 days after medium exchange with the standard, 459 and 522 mOsm kg^{-1} media (Fig. 3A). However, the LDH activity increased rapidly right after medium exchange with 580 mOsm kg^{-1} medium, suggesting that hyperosmotic pressure of 580 mOsm kg^{-1} induced cell lysis. The highest LDH activity (196 U l^{-1}) was observed in the standard medium at the end of culture because the dead cell concentration was highest in the standard medium.

In 14C6-bcl-2 cell cultures, the LDH activity was not significant in the standard and 459 mOsm kg^{-1} medium throughout the culture period (Fig. 3B). In addition, regardless of the media used, the LDH activity in 14C6-bcl-2 cell cultures was much lower than that in SH2-0.32-Δbcl-2 cell cultures, suggesting that overexpression of Bcl-2 improved the cellular membrane integrity under hyperosmotic pressure. Like SH2-0.32-Δbcl-2 cell culture, the LDH activity in 14C6-bcl-2 cell culture also increased right after medium exchange with 580 mOsm kg^{-1} medium, but at a much slower rate and to a lesser extent.

3.3. Quantitation of apoptosis and necrosis

To verify the cellular death mechanisms under the hyperosmotic pressure, nuclear morphology was characterized using AO–EB double fluorescence staining method as described in Section 2.

Fig. 4 shows the representatives of nuclear morphology; viable and non-apoptotic (VNA: bright-green nucleus and non-fragmented chromatin); viable and apoptotic (VA: bright-green nucleus and fragmented chromatin); non-viable and apoptotic (NVA: bright-orange nucleus and fragmented chromatin); non-viable and necrotic (NVN: bright-orange nucleus and non-fragmented chromatin). Although, the discrimination is distinct among the representatives shown in Fig. 4, we could not make accurate quantitation of VNA and VA cells because the chromatin morphology of VNA cells undergoing mitotic cell division (nuclear compaction) was similar to that of VA cells. Since VA cells were known to account for only a small proportion during apoptotic cell death probably due to the rapid loss of membrane integrity after chromatin fragmentation (Mercille and Massie, 1994), we did not distinguish VNA and VA cells in determining the proportion of cells undergoing apoptotic or necrotic cell death.

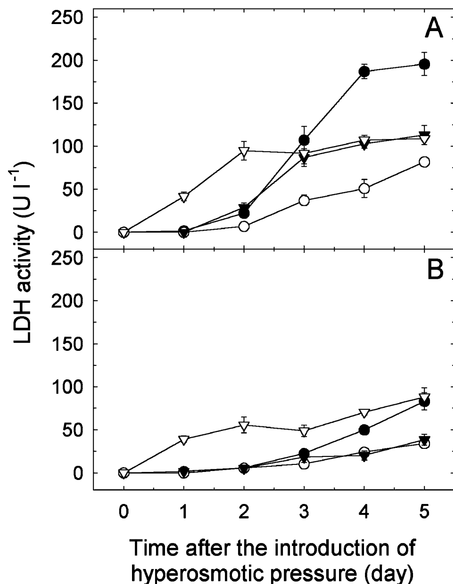

Fig. 3. Time-course changes in extracellular LDH activity in culture medium after medium exchange in: (A) SH2-0.32-Δbcl-2; and (B) 14C6-bcl-2 cell cultures. ●, 294 mOsm kg^{-1}; ○, 459 mOsm kg^{-1}; ▼, 522 mOsm kg^{-1}; ▽, 580 mOsm kg^{-1}. The error bars represent the standard deviations calculated from the data obtained in duplicate experiments.

Fig. 5 shows the changes in viability determined by counting the apoptotic and necrotic dead cells under the fluorescence microscope after the introduction of hyperosmotic pressure. The viability profiles were similar to those obtained by trypan blue dye exclusion method (Fig. 1).

In SH2-0.32-Δbcl-2 cell cultures, NVA cell population started to increase 2 days after medium exchange with the standard medium and accounted for approximately 35% of total cells 4 days after medium exchange (Fig. 5A). After medium exchange with 522 mOsm kg^{-1} medium, the NVA cell population gradually increased and was approximately 22% of total cell population 3 days after medium exchange. Except for the case of 580 mOsm kg^{-1} medium, most of dying cells underwent apoptosis and the cell population in necrotic cell death was less than 10%. After medium exchange with 580 mOsm kg^{-1} medium, the populations of both NVA and NVN cells increased significantly immediately after medium exchange. However, the necrotic cell death overwhelmed the apoptotic cell death 2 days after medium exchange.

In 14C6-bcl-2 cell cultures, the populations of NVA and NVN cells did not increase significantly in the standard, 459, and 522 mOsm kg^{-1} throughout the culture period, indicating that Bcl-2 overexpression suppressed apoptotic cell death (Fig. 5B). They accounted for more or less 10% of total cell population at the end of culture. In

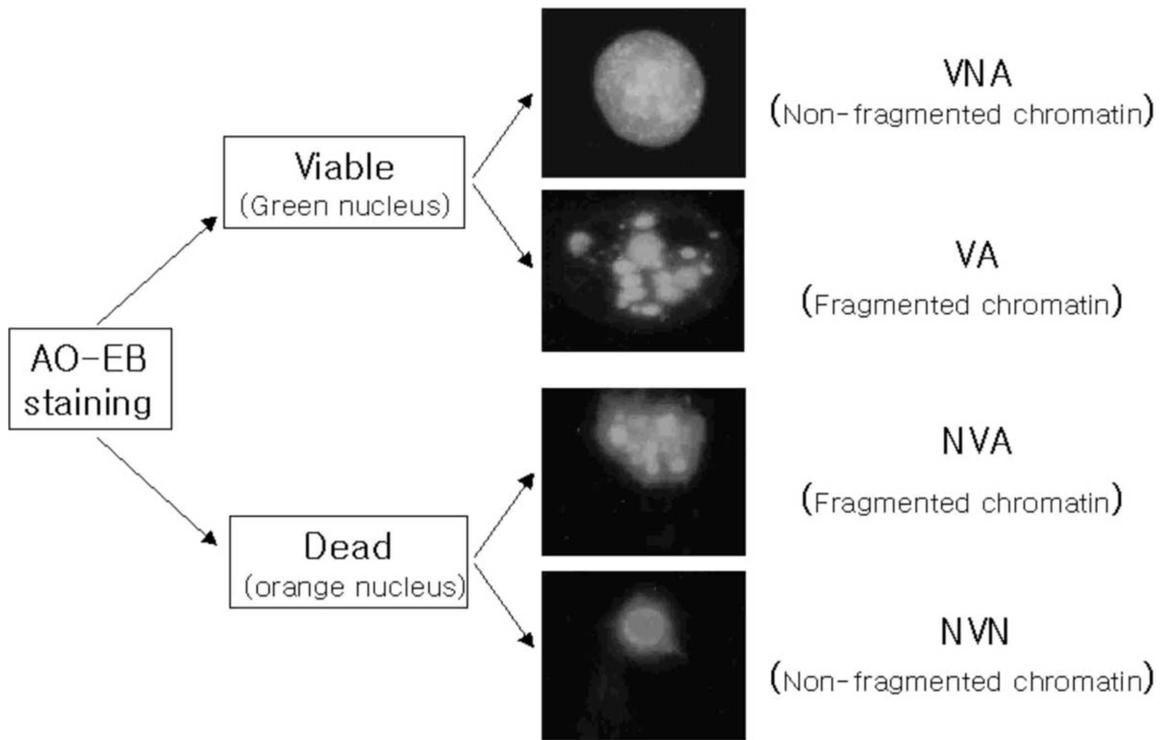

Fig. 4. Representatives of nuclear morphology characterized by AO–EB double staining method in two different cell death modes, apoptosis and necrosis. VNA, viable and non-apoptotic; VA, viable and apoptotic; NVA, non-viable and apoptotic; NVN, non-viable and necrotic.

contrast, it was noted that the populations of NVA and NVN cells increased immediately after medium exchange with 580 mOsm kg^{-1} medium and the NVN cell population undergoing the necrotic cell death was dominant.

3.4. Measurement of caspase-3 activity under hyperosmotic pressure

As shown in Fig. 5, Bcl-2 overexpression enabled 14C6-bcl-2 cells to be resistant against apoptosis induction in 522 mOsm kg^{-1}, but not in 580 mOsm kg^{-1}. To understand the inhibition mechanism of hyperosmolality-induced apoptosis by Bcl-2 overexpression, the changes in caspase-3 activity in SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells were measured by Western blot analysis.

Fig. 6 shows the changes in caspase-3 activity, which was measured by observing the cleavage of intracellular caspase-3 substrate, PARP protein, 1 and 2 days after medium exchange. In the standard, 459 and 522 mOsm kg^{-1} media, both SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells did not show remarkable degradation of PARP protein, 1 day after medium exchange. Only in 580 mOsm kg^{-1} medium, the cleavage of PARP protein was detected in both SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells, coinciding with rapid increase in NVA cell population (Fig. 5). On the other hand, 2 days after medium exchange with 522 mOsm kg^{-1} medium, the differences between SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells in regard to degradation of PARP protein could be observed. In case of SH2-0.32-Δbcl-2 cells, the clear sharp band of degraded PARP protein began to appear while it was not observed in 14C6-bcl-2 cells. Thus, it was demonstrated that hyperosmolality-induced apoptosis in rCHO cells is mediated in part by apoptosis-specific caspase-3, and Bcl-2 overexpression can delay its activation and thereby, inhibit the relatively

mild hyperosmolality-induced apoptosis (up to 522 mOsm kg^{-1} in this study).

4. Discussion

Hyperosmotic pressure, which can increase *q*, has been suggested as being an economical solution to increase the foreign protein production in rCHO cell cultures (Kim et al., 2000; Ryu et al., 2000). However, to adopt the process utilizing hyperosmotic pressure for improved foreign protein production in rCHO cell cultures, depressed cell growth at hyperosmolality should be overcome. To develop a strategy to overcome it, the effect of hyperosmotic pressure on the growth and death of rCHO cells, the most common host cells used for commercial production of therapeutic proteins, needs to be investigated substantially further. Although, many researchers take it for granted that hyperosmolar pressure can induce cell death over a certain range, the effect of hyperosmotic pressure on rCHO cell death has not been reported yet.

In this study, we investigated the cellular responses of rCHO cells to hyperosmotic pressure in regard to cell growth, antibody production, and particularly cell death. Furthermore, we demonstrated the potential of Bcl-2 overexpression in rCHO cells in hyperosmotic culture, which is known to be capable of providing the cells with resistance against apoptotic cell death in various stressful conditions.

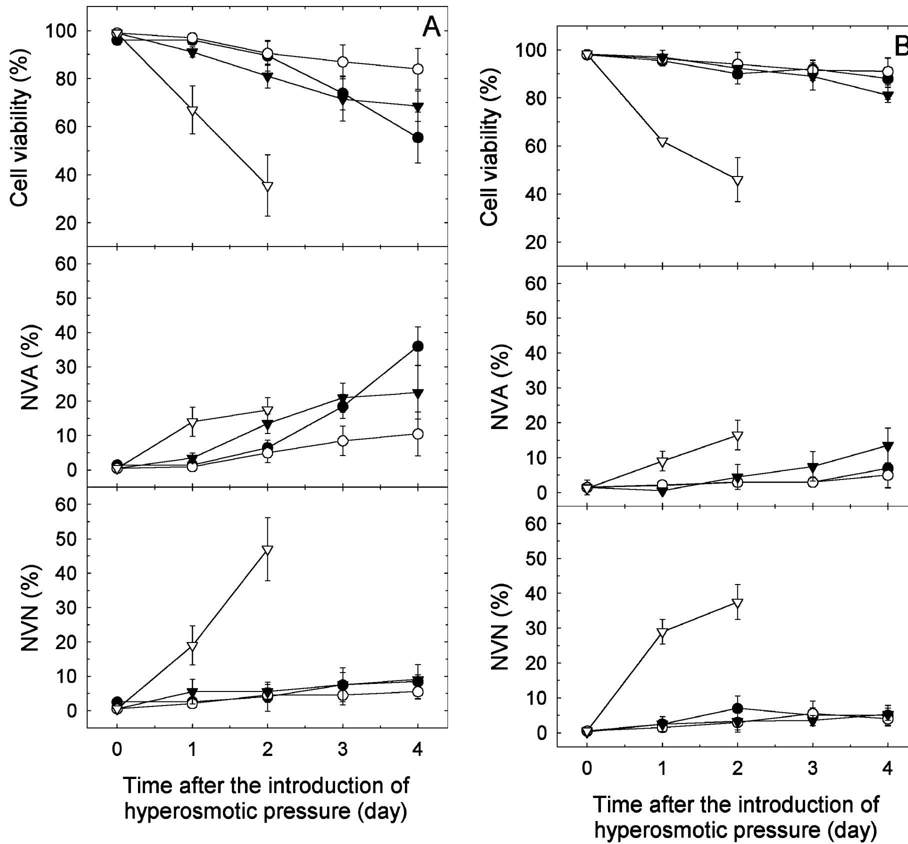

Fig. 5. Changes in viability and relative proportions of dead cells undergoing apoptosis or necrosis after the introduction of hyperosmotic pressure in: (A) SH2-0.32-Δbcl-2; and (B) 14C6-bcl-2 cell cultures. ●, 294 mOsm kg^{-1}; ○, 459 mOsm kg^{-1}; ▼, 522 mOsm kg^{-1}; ▽, 580 mOsm kg^{-1}. The relative proportions of apoptotic and necrotic cells were determined by AO–EB double staining methods. The error bars represent the standard deviations calculated from the data obtained in duplicate experiments.

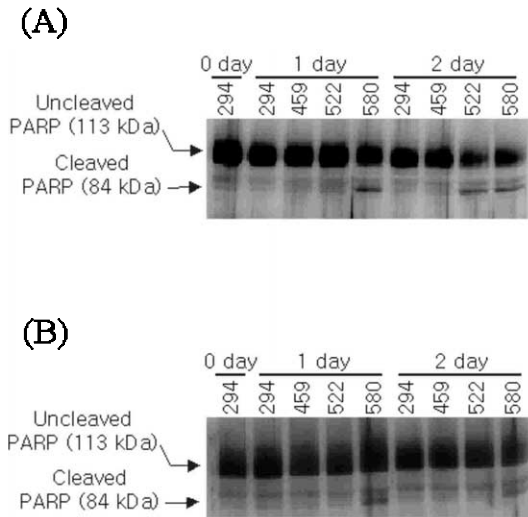

Fig. 6. Protective effect of Bcl-2 overexpression from PARP protein degradation triggered by active caspase-3 occurring in apoptotic cells. (A) SH2-0.32-Δbcl-2; and (B) 14C6-bcl-2 cells.

The inhibitory effect of hyperosmotic pressure was found to be dose-dependent (Fig. 1), though Bcl-2 overexpressing cells (14C6-bcl-2) were less susceptible to hyperosmotic pressure than control cells (SH2-0.32-Δbcl-2). At 459 mOsm kg^{-1}, the cytostatic effect of hyperosmotic pressure was observed in both SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells that displayed significantly reduced cell growth. However, despite the reduced cell growth and integral of viable cell concentration over culture time, the final antibody concentrations in SH2-0.32-Δbcl-2 and 14C6-bcl-2 cell cultures at 459 mOsm kg^{-1} were increased by 1.4- and 1.6-fold, respectively, compared with those at 294 mOsm kg^{-1} (physiological osmolality). This increase in final antibody concentration was achieved because the enhancement of q_{Ab} induced by hyperosmotic pressure outweighed the inhibitory effect of hyperosmotic pressure on cell growth.

Although, the detailed mechanism of enhanced q_{Ab} resulting from hyperosmotic pressure has not been clearly understood at the basic cellular level, various mechanisms were proposed to explain the enhanced q_{Ab} such as enhanced nutrient transport, increase in intracellular pH, G_1 cell cycle arrest, increased transcription and/or translation rates (Cherlet and Marc, 1999; Lee and Lee, 2000; Ryu et al., 2000).

At 522 mOsm kg^{-1}, the cytotoxic effect of hyperosmotic pressure began to appear in SH2-0.32-Δbcl-2 cells. After the failure in adaptation to 522 mOsm kg^{-1} hyperosmotic pressure during the first day after the introduction of hyperosmotic pressure, they underwent the gradual cell death mainly through the apoptotic cell death (Figs. 1 and 5). On the other hand, the apoptosis induced by 522 mOsm kg^{-1} hyperosmotic pressure could be delayed in 14C6-bcl-2 cells, which is probably due to the acquired apoptosis-resistance by overexpression of Bcl-2 survival protein. The delayed apoptosis induction was confirmed by the inhibition of caspase-3 activation (Fig. 6).

Caspase-3 is one of the major apoptotic effector caspases which activate the nuclear endonuclease and break down the critical cellular components and therefore, collapse the cellular functions. It was reported that cytochrome *c* (cyt *c*) release from mitochondria plays a pivotal role in the induction of caspase-9-mediated apoptosis by cytotoxic agent, and Bcl-2 protein localized in mitochondrial membrane can prevent cyt *c* efflux from mitochondria and formation of apoptosome in cytoplasm composed of cyt *c*, Apaf-1, and procaspase-9 (Green and Reed, 1998; Kluck et al., 1997; Thornberry and Lazebnik, 1998). Other researchers also reported that type I insulin-like growth factor (IGF-I) treatment or IGF-I receptor (IGF-IR) activation significantly inhibited the high mannitol concentration-induced apoptosis by maintaining the normal level of intracellular Bcl-2 protein and preventing protease activation, such as caspase-3, in neuroblastoma cells (Matthews et al., 1997; Singleton et al., 1996; Van Golen and Feldman, 2000). The resistance against apoptosis induced by 522 mOsm kg^{-1} hyperosmotic pressure in 14C6-bcl-2 cells improved the viable cell culture longevity with enhanced q_{Ab}, resulting in the highest final antibody concentration (Fig. 1).

At 580 mOsm kg^{-1} or higher, acute hyperosmotic pressure induced the rapid loss of viability in both SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells (about 50% within 24 h, Fig. 1) through necrosis rather than apoptosis (Fig. 5). As mentioned earlier, Bcl-2 overexpression could not improve the

viability in 14C6-bcl-2 cells because necrotic cell death could not be controlled by genetic modification. Thus, it was found that 14C6-bcl-2 cells could not tolerate the osmotic pressure of 580 mOsm kg^{-1}. However, since cell death mechanism induced by cytotoxic hyperosmotic pressure appears to be dependent on cell types, salts and the degree of osmotic shock (Leroy et al., 2000; Malek et al., 1998), the maximum, tolerable osmolality is likely to vary among rCHO cell lines.

The potential of Bcl-2 overexpression in hyperosmotic rCHO cell cultures for foreign protein production may also come from the downstream process where improved cell viability facilitates protein purification by reducing the unwanted proteases and glycosidases affecting the molecular integrity of foreign proteins. It was reported that the degree of product degradation and the extracellular proteolytic activity increased with the increasing LDH activity in spent medium with culture time (Cruz et al., 2000; Teige et al., 1994). In the range of 294–522 mOsm kg^{-1}, the improved cell viability and cell membrane integrity by Bcl-2 overexpression in 14C6-bcl-2 cells (Figs. 1 and 3) may be a good indicator of product quality produced in hyperosmotic rCHO cell cultures.

In conclusion, hyperosmotic pressure inhibited the growth of rCHO cells (SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells) in a dose-dependent manner while it increased q_{Ab}. The cytotoxic effect of hyperosmotic pressure began to appear at 522 mOsm kg^{-1} in SH2-0.32-Δbcl-2 cells that lost their viability mainly through apoptosis. Bcl-2 overexpression in 14C6-bcl-2 cells could delay the 522 mOsm kg^{-1} hyperosmolality-induced apoptosis by inhibiting caspase-3 activation. As a result, the medium exchange to 522 mOsm kg^{-1} hyperosmolar medium after 3 days of cultivation in 14C6-bcl-2 cell culture resulted in 2.5-fold increase in final antibody concentration, compared with the medium exchange to standard medium with physiological osmolality (294 mOsm kg^{-1}). At 580 mOsm kg^{-1}, Bcl-2 overexpression in 14C6-bcl-2 cells failed to improve cell viability because necrosis was the major cell death mode. Taken together, Bcl-2 overexpression and mild hyperosmolality (522 mOsm kg^{-1} in this study) can improve the antibody production of rCHO cells.

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