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Short communication

Effect of Bcl-xL overexpression on erythropoietin production in recombinant Chinese hamster ovary cells treated with dimethyl sulfoxide

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ABSTRACT

Dimethyl sulfoxide (DMSO) can increase the specific productivity (q) of foreign proteins in mammalian cells, while it can also induce cell death, particularly apoptosis. Bcl-xL is a typical anti-apoptotic protein that inhibits the apoptosis in recombinant Chinese hamster ovary (rCHO) cell culture. To evaluate the potential role of Bcl-xL overexpression on DMSO-mediated erythropoietin (EPO) production, we used EPO-producing rCHO cells with regulated Bcl-xL overexpression (EPO-off-Bcl-xL) by doxycycline. Although DMSO addition enhanced specific EPO productivity (q_{EPO}), it also induced cell death in EPO-off-Bcl-xL cells. Bcl-xL overexpression reduced the DMSO-induced cell death followed by release of various enzymes from plasma membrane-damaged cells as evidenced from LDH assay, resulting in delayed loss of EPO. However, it did not significantly improve the maximum EPO production. In addition, Bcl-xL overexpression suppressed DMSO-induced apoptosis, characterized by DNA fragmentation and Annexin V staining. Taken together, Bcl-xL overexpression could inhibit DMSO-induced apoptosis, thereby delaying the loss of EPO.

1. Introduction

Recombinant Chinese hamster ovary (rCHO) cells are one of the most popular host cells for the production of therapeutic proteins. For high-level protein production in rCHO cells, numerous strategies have been used to enhance specific protein productivity (q) and to increase integral of viable cell density [1]. The application of some q-enhancing factors to rCHO cells induces growth arrest and/or apoptosis. To improve the efficacy of q-enhancing factors in rCHO cell culture, they should be combined with additional methods to compensate for their detrimental effects on cell growth, e.g., sodium butyrate (NaBu) or hyperosmolality with an anti-apoptotic protein [2,3], and hyperosmolality with an osmoprotectant [4].

Dimethyl sulfoxide (DMSO) is frequently used as a solvent for water-insoluble compounds and as a cryoprotectant for mammalian cells [5]. Also, DMSO has been found to be a q-enhancing factor in rCHO cells [6,7]. DMSO-enhanced q may give rise to increased transcriptional activity, but not gene copy number or mRNA stability [8]. Similarly, the influence on CHO proteome by DMSO has been studied to figure out the intracellular response to the DMSO [9]. Despite its q-enhancing effect, a high concentration of DMSO may induce apoptosis in rCHO cells, e.g., DMSO-induced apoptosis in EL-4T lymphoma cells and SV40-transformed human keratinocytes [10,11]. As cell death, including apoptosis, affects product quantity as well as quality, the alleviation of DMSO-induced apoptosis enables the application of high concentrations of DMSO to rCHO cell cultures.

Cell engineering strategies dealing with anti-apoptotic proteins have been intensively investigated and are effective in rCHO cell culture [12–15]. Overexpression of Bcl-2 family proteins, Bcl-2 and Bcl-xL, showed a positive effect on culture longevity in rCHO cells via blocking programmed cell death (PCD) in batch culture as well as apoptosis (type I PCD) induced by NaBu and hyperosmolality [12,16]. Despite the potential relevance of Bcl-xL overexpression in inhibiting DMSO-induced cell death, Bcl-xL-overexpressing rCHO cells have not been used for DMSO-mediated protein production.

In this study, we investigated the effects of Bcl-xL overexpression on DMSO-mediated EPO production in rCHO cells. To exclude the possibility of clonal variability usually encountered in constitutive overexpression experiments, we used a controlled overexpression system that enables us to compare the same clone under different conditions.

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2. Materials and methods

2.1. Cell line, culture maintenance, and batch culture

The double-stable Tet-off CHO-EPO cells overexpressing Chinese hamster Bcl-xL (EPO-off-Bcl-xL) for inducible overexpression of Bcl-xL were established as described previously [15]. They were adapted to grow in suspension culture with a serum-free medium (HyQ SFM4CHO; HyClone, Logan, UT) supplemented with 4 mM glutamine (HyClone) and 100 ng/mL doxycycline (BD Biosciences Clontech, Palo Alto, CA).

Exponentially growing EPO-off-Bcl-xL cells were inoculated at a concentration of 2×10^5 cells/mL into 125-mL shake flasks (Corning, Corning, NY) containing 50 mL of the serum-free medium. The shake flasks were rotated at 110 rpm on an orbital shaker (Vision, Incheon, Korea), which was placed in a 5% CO_2/air incubator, humidified at 37 °C. For the control culture without Bcl-xL overexpression, doxycycline was added to the medium at 100 ng/mL every 3 days. Samples were taken at indicated times for the determination of viable cell concentration and viability.

2.2. Cell concentration, viability, and EPO assay

Cell concentration and viability were estimated with a Cytolecon automated cell imaging counter (CYT-100; ECI Inc., Japan) using the trypan blue dye exclusion method. The secreted EPO concentration was quantified using the Quantikine IVD ELISA Kit (R&D Systems, Minneapolis, MN).

2.3. Western blot analysis

Total cellular protein was analyzed by Western blot as described previously [16]. Antibodies used for Western blot were anti-Bcl-xL antibody (Cell Signaling Technology, Beverly, MA) and anti-β-actin antibody (Sigma, St. Louis, MO). After washing the blots with PBS-T buffer (phosphate buffered saline containing 0.1% Tween-20), bands were visualized using the ECL Western blotting system (Amersham, Uppsala, Sweden).

2.4. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity in the culture supernatant was measured using the Cytotoxicity Detection Kit^{PLUS} (Roche, Indianapolis, IN), as described previously [16].

2.5. DNA fragmentation assay by electrophoresis

Cells were harvested at designated time points during the batch culture. Intact and fragmented chromosomal DNAs were prepared from total cells (dead and viable cells), as described previously [16].

2.6. Annexin V/propidium iodide staining assay

The Annexin V/propidium iodide (PI) staining assay was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences Clontech) according to the manufacturer's instructions. 1×10^5 cells were harvested at indicated time points during batch culture with DMSO addition. Cells were then incubated with FITC-labeled Annexin V and PI for 15 min at room temperature in the dark, followed by adding assay buffer prior to flow cytometric analysis. Flow cytometric analysis was performed using the FACSAria^{TM} System (BD Biosciences Clontech). The *X*-axis plots the fluorescence of FITC Annexin V (green) while the *Y*-axis plots the fluorescence of PI (red). Quadrant setting was based on the control sample that is not stained with FITC-labeled Annexin V and PI. The percentage of cell population in different quadrants was calculated using quadrant statistics.

3. Results and discussion

Clonal variability is an important issue in investigating the effect of gene expression in rCHO cells; the application of a controlled overexpression system helps to avoid this issue. EPO-producing rCHO cells with Bcl-xL expression (EPO-off-Bcl-xL) regulated by the Tet-off system was employed in the present study [16]. Doxycycline was used to regulate Bcl-xL expression owing to its stability. Bcl-xL expression in EPO-off-Bcl-xL cells is completely repressed at a doxycycline concentration of 100 ng/mL, and this concentration does not alter cell growth or protein production [16]. To determine whether Bcl-xL expression in EPO-off-Bcl-xL cells was maintained after 20 passages without selective pressure, including Zeocin and hygromycin, the expression levels of Bcl-xL over time was assessed by Western blot analysis. Fig. 1 shows that Bcl-xL overexpression

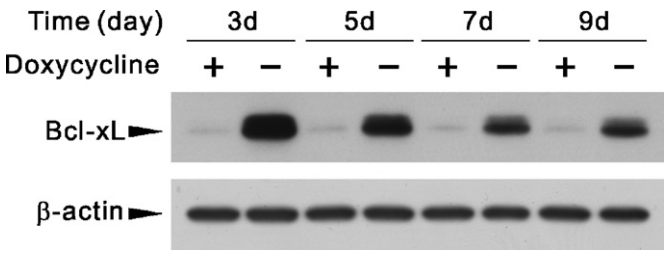

Fig. 1. Inducible overexpression of Bcl-xL regulated by doxycycline in EPO-off-Bcl-xL cells. The double-stable Tet-off CHO-EPO cells overexpressing Chinese hamster Bcl-xL (EPO-off-Bcl-xL) regulated by doxycycline was inoculated at 2×10^5 cells/mL into 125-mL shake flasks containing 50 mL of serum-free medium. Doxycycline was added every 3 days. Total cellular protein was analyzed by Western blot. Equal loading of cells was verified by Western blot analysis with an anti-β-actin antibody.

was tightly regulated by doxycycline, although its expression level was reduced at the end of the batch culture.

To evaluate the effects of Bcl-xL overexpression on cell growth and EPO production in EPO-off-Bcl-xL cells treated with DMSO, cells were cultivated in the presence or absence of doxycycline. After 4 days of cultivation, DMSO was added to the culture medium at the final concentration of 3% which was chosen for considering the cell death and specific EPO productivity (q_{EPO}) (data not shown). As a control, cells were also cultured in the presence of doxycycline without DMSO.

Fig. 2 shows the typical cell growth, viability, and EPO production profiles during batch culture. DMSO suppressed cell growth, consistent with previous studies in rCHO cells [6,8]. Regardless of Bcl-xL overexpression, the maximum viable cell concentration under DMSO treatment was found to be 65% of the control culture. Similarly, Bcl-xL overexpression did not significantly affect maximum viable cell concentration in the absence of DMSO (data not shown). However, Bcl-xL overexpression significantly improved the DMSO-decreased cell viability. Cell viability on day 9 in the presence of doxycycline (without Bcl-xL overexpression) under DMSO treatment was $45.3 \pm 1.6\%$, while it increased to $76.0 \pm 2.3\%$ in the absence of doxycycline (with Bcl-xL overexpression). As expected from previous reports [6,8], DMSO addition enhanced q_{EPO}, EPO production rate per cell. q_{EPO} was calculated from data showing a linear correlation between the EPO concentration and time integral of viable cells after day 4 [17]. q_{EPO} in the control culture was $1.9 \pm 0.2\ \mu g/10^6$ cells/day, while it increased to $3.5 \pm 0.4\ \mu g/10^6$ cells/day without Bcl-xL overexpression and $3.0 \pm 0.2\ \mu g/10^6$ cells/day with Bcl-xL overexpression under DMSO treatment, respectively. Despite the increased q_{EPO}, the maximum EPO concentration was not significantly increased by DMSO; however, Bcl-xL overexpression delayed the loss of EPO at the end of batch culture.

The degree of cell death can be quantified by measuring the activity of released lactate dehydrogenase (LDH) from plasma membrane-damaged cells in the culture medium [18]. We assessed LDH activity to confirm the effect of Bcl-xL overexpression on cell death in cultures with DMSO (Fig. 2). Consistent with the changes in cell viability, LDH activity rapidly increased after the addition of DMSO. Although the LDH activity in cells overexpressing Bcl-xL started to increase after DMSO addition, its rate of increase was much lower than in the absence of Bcl-xL overexpression. Taken together, Bcl-xL overexpression reduces the degree of cell death including plasma membrane-damaged cells induced by DMSO, and this may be a reason for the reduction in loss of EPO.

Nucleosomal DNA fragmentation is a well-characterized hallmark of apoptosis, and it is commonly used to monitor apoptosis [19]. To determine whether Bcl-xL overexpression affects DMSO-induced apoptosis during batch culture, we assessed DNA fragmentation. Fig. 3 shows the changes in the degree of DNA frag-