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Expression of anti-apoptotic *30Kc6* gene inhibiting hyperosmotic pressure-induced apoptosis in antibody-producing Chinese hamster ovary cells

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ABSTRACT

In mammalian cell culture, elevating osmotic pressure can improve recombinant protein production by increasing the specific productivity. However, this operation also induces cell apoptosis. Thus, its beneficial effect is compromised. Previously, the expression of the *30Kc6* gene was found to inhibit apoptosis in Chinese hamster ovary (CHO) cells, resulting in an increase in recombinant protein production. In this study, the *30Kc6* gene was introduced into an antibody-producing CHO cell line, and its effect on hyperosmotic pressure-induced apoptosis was investigated. In the standard medium, the expression of *30Kc6* increased cell viability by 34.1% and productivity to 2.3 folds. After the osmotic pressure shift to 410 mOsm/kg, it was found that the viability of the *30Kc6*-expressing cell decreased only by 8.5% as compared with that of the standard culture, while it decreased by 27.1% for the control cell. Consequently, the maximum production of the *30Kc6*-expressing cell increased to 3.8 folds relative to that of the control cell in the standard condition. However the production rate did not increase for the control cell under the same conditions. *30Kc6* expression inhibited the hyperosmotic pressure-induced apoptosis at least partially because it repressed the mitochondrial membrane potential (MMP) reduction.

1. Introduction

In mammalian cell culture, increasing the specific productivity (q) is a widely used strategy for improving recombinant protein production. There are two general approaches to increase q. One is genetic engineering method through over-expressing *disulfide isomerase* (PDI) [1,2], *TorsinA* [3], *calnexin* and *calreticulin* [4], among others. The other one is process design, such as decreasing culture temperature [5], adding sodium butyrate [6] and elevating osmotic pressure [7–11]. In terms of the osmotic pressure elevation, addition of 90 mM NaCl to the production medium of transient antibody production in Chinese hamster ovary (CHO) cells increased the volumetric yield up to 4 folds [7]. However, for the stable hybridoma [8,9] and CHO cells [10,11], although q was also enhanced, the final product concentration was not increased, because the hyperosmotic pressure severely induced cell death including apoptosis and necrosis in a dose-dependent manner, and its beneficial effect was compromised [12].

To overcome this drawback, a biphasic culture strategy was adopted (osmotic pressure shift) [13,14]. In brief, the cells were first cultivated in the standard medium with physiological osmolality until the cell density reached a relative higher level, the osmotic pressure was then elevated. By this means, the maximum antibody production increased by 161% [13]. To further enhance recombinant protein production under the hyperosmotic condition, the anti-apoptotic genes, such as *Bcl-2* and *Bcl-x_L*, were over-expressed [12,15]. After the osmotic pressure shift from the standard medium (294 mOsm/kg) to 522 mOsm/kg, *Bcl-2* over-expression significantly inhibited cell apoptosis, resulting in a 2.5-fold increase in antibody production [12]. Through simultaneously applying of *Bcl-x_L* over-expression and repeated nutrient feeding (this induced a gradual increase in osmotic pressure), the EPO production increased by 1.87 folds [15].

Previously, the silkworm hemolymph was reported to inhibit apoptosis in both insect and human cells [16,17]. The anti-apoptotic components were found to be a group of structurally related proteins termed 30 K proteins including 30Kc6, 30Kc12, 30Kc19, 30Kc21 and 30Kc23 with molecular weights of approximately 30 kDa [18]. However, among the five homologues, only the 30Kc6 protein exhibited potent anti-apoptotic activity [19]. Hence, to improve recombinant protein production, the anti-apoptotic *30Kc6* gene was expressed in CHO cells producing EPO [20]. As a consequence, the cell density and productivity increased by 5 and 10 folds, respectively. To date, the anti-apoptotic mechanism of 30Kc6 protein is not fully revealed. Our unpublished data showed that the silkworm hemolymph did not inhibit the apoptosis of Bax-deficient Jurkat cells induced by staurosporine, suggesting that the

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30Kc6 protein might repress the Bax-mediated apoptotic signaling pathway.

In this study, to inhibit the hyperosmotic pressure-induced apoptosis for improving antibody production, the anti-apoptotic *30Kc6* gene was expressed in a CHO cell line producing a chimeric anti-human CD20 monoclonal antibody (mAb). The results show that the expression of *30Kc6* inhibited hyperosmotic pressure-induced apoptosis, and achieved a significant increase in antibody production.

2. Materials and methods

2.1. Cell line, transfection and selection of 30Kc6-containing cells

The host cell, a CHO cell line producing a chimeric anti-human CD20 monoclonal antibody, was kindly provided by Prof. Beifen Shen (Academy of Military Medical Sciences, China). This cell line was generated using the methotrexate-mediated dihydrofolate reductase (DHFR) amplification system. The adherent cells were maintained in DMEM supplemented with 10% (v/v) dialyzed fetal bovine serum (dFBS; Gibco, Grand Island, NY). The pIRESHyg2-*FLAG-30Kc6* and pIRESHyg2 plasmids were a generous gift from Prof. Tai Hyun Park (Seoul National University, Korea). The *30Kc6* gene (GenBank accession number, X54735) was amplified by RT-PCR from fifth larval fat body RNA [21]. The *FLAG*-tagged *30Kc6* gene was inserted into the pIRESHyg2 plasmid at restriction sites of BamHI and NotI. The transfection was performed using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All the stable cell lines were selected by limiting dilution under the selective pressure of hygromycin (Sigma, St. Louis, MO). To avoid the clonal variability, three stable cell lines with different expression levels of 30Kc6 protein from 960 clones were established. Similarly, three negative controls with the plasmid alone were established at the same time.

2.2. Western blot analysis

1×10^6 cells in exponential phase were lysed in a radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 0.1% sodium dodecyl sulfate (SDS) and 1% protease inhibitor cocktail). The total protein content was quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Cramlington, UK). The samples were run on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane using a Bio-Rad Trans Blot SD Semidry Transfer Cell. A rabbit anti-FLAG polyclonal antibody (Cell Signaling, Beverly, MA) was used as the primary antibody for detecting the expression of 30Kc6 protein. The blots were probed with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody. Enhanced chemiluminescence (ECL) western blot detection reagents (GE Healthcare, Amersham, UK) were used to visualize the immunoreactive bands.

2.3. Cell culture, viability, viable cell density and ELISA assay

The adherent cells were inoculated into a 150 cm^2 T-Flask (Techno Plastic Products, Trasadingen, Switzerland) with 50 mL serum-free medium (Hyclone, South Logan, UT) supplemented with 4 mM of glutamine, and then placed on a rotary shaker with an agitation rate of 40 rpm in a humidified Thermo Scientific Forma® Series II Water Jacketed CO_2 Incubator (37 °C, 5% CO_2; Thermal Fisher Scientific, Waltham, MA). After several passages, the cells were completely adapted to suspension culture. The suspended cells at a cell density of 2×10^5 cells/mL were inoculated and cultivated in a fresh T-Flask (150 cm^2, Techno Plastic Products) under the same conditions. After 2 days of cultivation, the osmotic pressure was adjusted by adding 5 M NaCl. The osmotic pressure was measured using an auto freezing point osmometer (Shanghai Medical University Instrument Factory, Shanghai, China).

Cell density was determined using a hemacytometer. Viable cells were distinguished from dead cells using the trypan blue dye exclusion method.

The concentration of mAb secreted into the medium was measured using ELISA. Affinity purified goat antibody to human IgG (H + L) (Pierce, Rockford, IL) at a concentration of 4 μg/mL was used as capture antibody, and goat antibody to human IgG (H + L) conjugated with HRP (Pierce) at a 1:15,000 dilution was used as detection antibody. One percent bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.05% of Tween 20 was used for blocking. Tetramethyl benzidine (TMB) solution (Thermo Scientific, Cramlington, UK) was used as the substrate for color formation. The absorbance at 450 nm was recorded. The standard curve was prepared using serial dilutions of human affinity purified IgG (Sigma).

2.4. Evaluation of specific mAb productivity

The specific mAb productivity (q) was based on the data collected during the exponential phase (days 2–4) and was calculated by taking the slope of the antibody production against the time integral of viable cells [22].

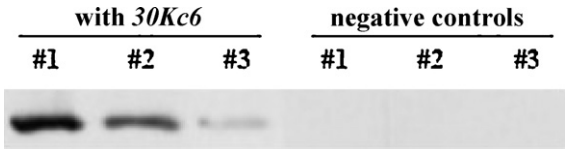

Fig. 1. Western blot analysis of the 30Kc6 protein using an anti-FLAG polyclonal antibody.

2.5. Flow cytometry analysis

1×10^6 cells were collected and resuspended in 400 μL of annexin V binding buffer containing fluorescein isothiocyanate (FITC)-conjugated annexin V (Mbchem, Shanghai, China). After 15 min of incubation, 10 μL of propidium iodide (PI, 25 μg/mL) was added. The cell suspension was immediately analyzed using fluorescence activated cell sorter (FACS) Calibur flow cytometer (BD Biosciences, San Jose, CA). The excitation wavelength was set at 488 nm with the emission wavelength set at 513 and 675 nm for detecting FITC-annexin V (FL1 channel) and PI (FL3 channel), respectively.

2.6. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was measured using 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1, Sigma). In brief, 5×10^5 cells were collected and resuspended in 1 mL PBS followed by addition of JC-1 to a final concentration of 0.5 μg/mL. The red (590 nm) and green (535 nm) fluorescence were measured using a spectrofluorometer (TECAN, Salzburg, Austria) with an excitation wavelength set to 485 nm. The ratio of red to green fluorescence was used to evaluate the MMP.

3. Results

3.1. Enhanced mAb productivity by the expression of 30Kc6 gene in suspension culture

In this study, three stable cell lines with different expression levels of 30Kc6 protein and three negative controls with vector alone were established (Fig. 1). Subsequently, these clones were adapted to suspension culture. In this process, the potent *30Kc6*-expressing clone #1 encountered growing difficulties. The cause of the slow growth could be due to a severe metabolic burden brought on by the excessive expression of the 30Kc6 protein in the host cell. Other clones that had completely been adapted to suspension culture were inoculated into fresh T-Flask. As shown in Fig. 2A and B, compared with the negative control clones, the *30Kc6*-expressing clones maintained significantly higher cell viabilities and viable cell densities. On day 7, the viability of the *30Kc6*-expressing clone #2 and #3 was 34.1 ± 5.0% ($P = 0.0005$, $n = 3$) and 17.5 ± 11.1% ($P = 0.03$, $n = 3$) respectively higher than that of the negative clone #1, whose viability was the highest among the negative controls. Also, it was noticed that the *30Kc6*-expressing clone #2, with higher expression of the 30Kc6 protein, showed relatively higher viability than the *30Kc6*-expressing clone #3. Similar variations in cell viability were also observed among the three negative clones. Moreover, the mAb productivity was also determined. As shown in Fig. 2C, the productivity of the *30Kc6*-expressing clone #2 and #3 increased to 2.3 and 1.7 folds relative to that of the negative clone #1 on day 5, respectively. These results indicated that the expression of *30Kc6* inhibited cell apoptosis and consequently increased mAb productivity in suspension culture.

3.2. Inhibiting hyperosmotic pressure-induced apoptosis and consequently enhancing mAb productivity by the expression of 30Kc6 gene

The effect of *30Kc6* expression on hyperosmotic pressure-induced apoptosis was investigated. First, the *30Kc6*-expressing clone #2 and the negative clone #1, which showed the highest cell viability and productivity, were selected to inoculate the