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Enhancement of human erythropoietin production in Chinese hamster ovary cells through supplementation of 30Kc19-30Kc6 fusion protein

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ABSTRACT

The importance of anti-apoptosis in mammalian cell culture has been widely recognized. We reported previously that expression of *Bombyx mori* 30 K genes in Chinese hamster ovary (CHO) cells increases recombinant protein production by inhibiting apoptosis and enhancing specific productivity. However, previous studies have shown expression of the anti-apoptotic 30Kc6 protein as inclusion bodies in *Escherichia coli*. 30Kc19 protein, another silkworm hemolymph protein, has cell-penetrating and recombinant protein productivity-improving properties, and we found that it improves soluble expression of its partner. In this study, we fused 30Kc6 with 30Kc19 as an expression partner to express a soluble fused protein and to deliver the protein to cells. Supplementing the recombinant 30Kc19-30Kc6 fusion protein in cell culture medium increased viability, effectively penetrated the cells, and inhibited CHO cell apoptosis by 68%. Moreover, the mitochondrial membrane potential and ATP generation also increased by 50% and 33%, respectively. Erythropoietin (EPO) productivity increased by > 30% because of the anti-apoptotic effect and increased specific productivity. These results demonstrate the potential use of this fusion protein as a supplement in mammalian cell culture during production of biopharmaceutical proteins.

1. Introduction

The Chinese hamster ovary (CHO) cell line has been widely used as a mammalian host cell [1,2] for producing recombinant glycoproteins, such as interferon-β, monoclonal antibodies, and erythropoietin (EPO) [3–5] because of its advantageous properties such as high production; suitability for large-scale culture; and similarities in the glycan structures produced from CHO cells to those of human glycoproteins [6–8]. However, there is a difficulty in maintaining high cell growth and specific productivity because cells are subjected to programmed cell death (PCD). This is a common problem encountered in cell culture; which decreases recombinant protein productivity. During the culture process, nutrient depletion, hypoxia, waste by-product accumulation, and other factors lead to cell apoptosis [9–11]. Various attempts have been performed to inhibit apoptosis, such as nutrient supplements [12], adding apoptosis inhibitors [13–16], and expressing anti-apoptotic genes [17,18]. Traditional methods, such as addition of sodium butyrate have been reported to increase recombinant protein production [19–21]. However, it also induces apoptosis by inhibiting histone deacetylation [22,23], which reduces cell viability and productivity. Thus, despite the beneficial effect on protein expression; the use of sodium butyrate is compromised by its cytotoxic effect.

Silkworm hemolymph consists of a group of structurally related proteins with a molecular weight of approximately 30 kDa, including 30Kc6, 30Kc12, 30Kc19, 30Kc21, and 30Kc23. During the fifth instar larval to early pupal stages, these "30 K proteins" are synthesized in fat body cells and accumulated in the hemolymph [24,25]. During metamorphosis from larva to pupa, these proteins are

Abbreviations: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle medium; *E. coli*, *Escherichia coli*; EPO, erythropoietin; HRP, horseradish peroxidase; PAGE, polyacryl amide gel electrophoresis; SDS, sodium dodecyl sulfate.

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transferred from the hemolymph to fat body cells and are deposited there until use [26,27]. We have demonstrated previously that silkworm hemolymph and 30 K proteins exhibit an anti-apoptotic effect in various cells by adding the protein to culture medium or by gene expression [18,28–39]. 30 K proteins also enhance production of recombinant EPO, interferon-β, and monoclonal antibodies; increase glycosylation, cell growth, and viability in various cells; and have an enzyme-stabilizing effect [17,40–46]. Additionally, the 30Kc19 protein has a cell-penetrating property when added to culture medium; thus, it can be applied to deliver cargo proteins, as it can penetrate cell membranes and stabilize cargo proteins [47,48].

In this study, soluble expression and purification of a 30Kc19-30Kc6 fusion protein in *Escherichia coli* was used for inhibiting apoptosis and improving recombinant protein production in CHO cells. Considering that EPO is one of the most demanded recombinant proteins, we added the 30Kc19-30Kc6 fusion protein to CHO cell culture medium to increase productivity of recombinant human EPO (rHuEPO) in serum-free media. This 30Kc19-30Kc6 fusion protein has advantageous properties of both 30Kc19 and 30Kc6, which are soluble, cell-penetrating, anti-apoptotic, and productivity-enhancing properties. This work demonstrates the potential use of this multifunctional protein in mammalian cell culture for the production of recombinant proteins.

2. Materials and methods

2.1. Construction of expression vectors

pET-23a/30Kc6 and pET-23a/30Kc19 plasmids were obtained from previous studies [35]; [48]. The forward primer 5′-GGA TCC GCA GAT TCC GAC GTC–3′ including the *Bam*HI restriction enzyme site and the reverse primer 5′-GAA TTC TGC TTT TGC TGC TGC TTC TTT TGC TGC TGC TTC TGC GAA AGC CTT TAT ACC-3′ with the *Eco*RI restriction enzyme site were used to construct the pET-23a/*30Kc19-30Kc6* plasmid. A linker (AEAAAKEAAAKA), as shown by the underline, was inserted between *30Kc19* and *30Kc6*, so that the two proteins did not interfere with each another [49] (Fig. 1C).

2.2. Protein expression and purification

The constructed vector was transformed into *E. coli* Rosetta gami (Novagen, Gibbstown, NJ, USA) and cells were grown in LB-ampicillin medium at 37 °C in a 200 rpm shaking incubator. Isopropyl 1-thio-β-D-galactopyranoside (1 mM) was used for induction, and all proteins were further incubated at 37 °C to produce protein, except 30Kc19-30Kc6, for which 27 °C was selected as the induction temperature. After centrifugation, the cells were harvested, resuspended in 20 mM Tris–HCl buffer (pH 8.0), and disrupted by sonication. All recombinant proteins were purified from the supernatant following cell lysis using a HisTrap HP column (GE Healthcare, Milwaukee, WI, USA), dialyzed against DMEM (Hyclone, Logan, UT, USA) or SFM4CHO-Utility (Hyclone) using a HiTrap Desalting column (GE Healthcare) with purity > 90% (data not shown), and stored at −70 °C until use. The protein quantitative analysis was performed using a Micro BCA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

All SDS-PAGE was conducted using 12% polyacrylamide gels. Samples were mixed with reducing sample buffer containing SDS and β-mercaptoethanol (pH 6.8), and were denatured by boiling. Each sample was separated according to size by electrophoresis. The polyacrylamide gel was immersed in Coomassie Blue staining solution and then immersed in destaining solution for analysis.

2.4. Cell line, cell culture, viability, viable cell density, and enzyme-linked immunosorbent assay (ELISA)

CHO cells producing rHuEPO, obtained from a previous study [17], were seeded on 12-well multiplates and maintained in a humidified atmosphere of 5% CO_2 at 37 °C in IMEM (Hyclone) supplemented with 10% (w/v) FBS. Culture media was replaced with serum-free media to produce EPO; SFM4CHO-utility (Hyclone). A 6.5 μM concentration of 30Kc19 or 30Kc19-30Kc6 protein was added to the culture medium and incubated for 12 h at 37 °C in a humidified atmosphere of 5% CO_2.

Cell density was calculated using a hemacytometer. The trypan blue dye exclusion method was used to distinguish viable from dead cells. The EPO secreted into the medium was measured using a Quantikine IVD EPO ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.5. Immunocytochemistry analysis

Cell penetration of the protein was visualized using confocal microscopy (Nikon, Tokyo, Japan). Cells were seeded on 8-well chamber slides (Nunc Lab-Tek, Rochester, NY, USA) and incubated overnight. Protein was added to the culture medium and incubated for 12 h at 37 °C in a humidified atmosphere of 5% CO_2. After the incubation, the cells were washed three times with PBS, fixed in 4% paraformaldehyde for 10 min, and incubated for 10 min with 0.25% Triton X-100 in PBS for permeabilization. The fixed cells were blocked with 5% bovine serum albumin (BSA) in 0.1% PBS-T for 1 h and then incubated with anti-T7 tag rabbit antibody (Abcam, Cambridge, MA, USA; 1:500 diluted in 1% BSA in 0.1% PBS-T). Alexa Fluor® 488 Goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA; 1:1000 diluted in 1% BSA in 1% PBS-T) was used for the secondary antibody. Cell nuclei were stained with Hoechst 33342 (Sigma, St. Louis, MO, USA) for 10 min. A confocal laser microscope (EZ-C1, Nikon) was used to observe intracellular fluorescence, and images were captured using the manufacturer's software (Nikon).

2.6. Immunoblot analysis

Cells were treated with trypsin-EDTA (Sigma) then washed three times with PBS to distinguish between intracellular and membrane-bound proteins. The collected cells were washed three times in PBS, and cell extracts were collected in RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail) at 4 °C for 1 h followed by centrifugation. Each cell extract containing an equal amount of protein was resolved by PAGE and examined by immunoblot analysis. Intracellular protein was detected using anti-T7 tag rabbit antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Millipore, Milford, MA, USA).

2.7. Apoptosis induction

CHO cells were seeded on 6-well multiplates at 2×10^4 cells/cm^2 and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Three days after seeding the CHO cells, apoptosis was induced by replacing the defined FBS-containing culture media with serum-free media containing 6.5 μM of 30Kc19 or 30Kc19-30Kc6 protein.

2.8. Flow cytometric analysis

The FITC Annexin V Apoptosis Detection Kit I (BD BioScience, San Jose, CA, USA) was used for flow cytometry. Cells (1×10^6)