Expression and Purification of Recombinant Human Angiopoietin-2 and Its Analog in Chinese Hamster Ovary Cells

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ABSTRACT

Angiopoietin-2 (Ang2) is a naturally occurring antagonist for angiopoietin-1 (Ang1) and its Tie2 receptor during vasculogenesis. Although angiopoietins have been expressed in several mammalian cell lines, their expression levels are low. Recombinant Chinese hamster ovary (CHO) cell lines expressing a high level of human Ang2 or its analog, human $Ang2_{443}$, with an amino-terminal FLAG-tag were constructed by transfecting the expression vectors into dhfr-deficient CHO cells and subsequent gene amplification in medium containing stepwise increments in methotrexate level. Secreted Ang2 or human $Ang2_{443}$ were purified from the cultured medium using an anti-FLAG-agarose affinity chromatography. The purified Ang2 and $Ang2_{443}$ migrated on SDS-PAGE as a broad band, characteristic of glycosylated protein. Their biological activity *in vitro* was demonstrated in a serum deprivation-induced apoptosis assay. Ang2 at high concentration, like Ang1, can be an apoptosis survival factor for endothelial cells through the activation of the Tie2 receptor.

INTRODUCTION

Angiogenesis is the process of generating new capillary blood vessels. It plays an important role in embryogenesis, wound healing, tumorigenesis, and the female reproductive system. Amongst the numerous regulators, vascular endothelial growth factor (VEGF) and angiopoietin families have been shown to play specific roles in angiogenesis due to the restricted expression of their receptors[1,2]. Out of the four currently known Angs (Ang1 to Ang4), Ang1 and Ang2 are the best characterized. Ang1 and Ang2 share about 60% amino acid identity and bind with similar affinity to the endothelial cell tyrosine kinase receptor, Tie2. Both Ang1 and Ang2 have characteristic protein structures, which form dimers and oligomers. Ang2 is a naturally occurring antagonist of Ang1 that competes for binding to the Tie2 receptor and blocks Ang1-induced Tie2 autophosphorylation during vasculogenesis. Recently, a cDNA encoding a shorter form of Ang2 was isolated from human umbilical vein endothelial cell cDNA , which designated as $Ang2_{443}$ because it contains 443 amino acids[3].

With an increasing interest in Angs expression in mammalian cells, there are reports of Angs

expression in COS cells, human embryonic kidney 293T cells, rat aortic A10 smooth muscle cells and HT29 human colon cancer cells, but all these expressions were transient. Here, we report the high-level, stable expression of rhAg2 and rhAng2$_{443}$ with an amino-terminal FLAG-tag in CHO cells. The FLAG-tagged rhAng2 and rhAng2$_{443}$ were purified from the culture medium of recombinant CHO cells using the anti-FLAG affinity gel. Finally, we demonstrate their biological activity *in vitro* in a binding assay to Tie2, in a phospholylation assay of Tie2 receptor, and in a serum deprivation-induced apoptosis assay.

MATERIALS AND METHODS

Construction of expression vectors The rhAng2 expression vector (pAng2-dhfr) was constructed by modifying pFLAG-hAng2 and pKc-dhfr-hus plasmids (Fig. 1). A cDNA fragment of pFLAG-hAng2 contains rhAng2 with an amino-terminal FLAG and a preprotrypsin leader instead of native signal sequence for secretion. It was inserted into the Eco RV site of pcDNA3.1/ZEO(+) and then digested with Apa I/ Mlu I to insert into pKc-dfhr-hus previously digested with Apa I/ Mlu I. For high-level expression, the transcription of rhAng2 cDNA is controlled by cytomegalovirus (CMV)/enhancer. The rhAng2$_{443}$ expression vector (pAng2$_{443}$-dhfr) was constructed in the same manner by modifying pFLAG-hAng2$_{443}$ and pKc-dhfr-hus plasmids.

Vector transfection, and cell line development CHO/*dhfr*$^-$ cells (CRL-9096; ATCC) were grown and maintained in IMDM supplemented with 10^{-4} *M* hypoxanthine and 10% FBS. CHO/*dhfr*$^-$ cells were transfected with 2 μg of the pAng2-dhfr or pAng2$_{443}$-dhfr construct using LipofectAMINE^{TM} reagent. Drug selection was carried out by seeding 10^3 cells/well in 96-well tissue culture plates containing IMDM supplemented with 10% dFBS and 500 μg/mL G418. G418 was used only in this first selection. The culture supernatant of G418-resistant clones was tested for rhAng2 and rhAng2$_{443}$ production by an enzyme linked immunosorbent assay.

Among G418 resistant clones, 55 clones producing rhAng2 and 87 clones producing rhAng2$_{443}$ were transferred to 96 well culture plates for estimation of relative angiopoietin productivity. When the cells reached confluency, the supernatant was completely removed from each well and saved for rhAng2 and rhAng2$_{443}$ assay. The concentration of viable cells remained in each well was estimated using MTT assay[4]. The relative angiopoietin productivity per cell (q_{Ang}) of each clone was determined by dividing the angiopoietin concentration in the supernatant by the corresponding OD value (OD_{570}-OD_{630}) from the MTT assay. Based on the their relative q_{Ang}, 10 high producing clones were selected for gene amplification and were subjected to increasing levels of methotrexate (0.02, 0.08, and 0.32 μ*M*).

Purification of rhAng2 in CHO cells Cells amplified at 0.08 μ*M* MTX (Ang2-3-0.08 for rhAg2 production and A2$_{443}$-4-0.08 for rhAng2$_{443}$ production) were seeded at 2 × 10^5 cells/mL in T-175cm^2 containing 35 mL of IMDM supplemented with 10% dFBS and 0.32 μ*M* MTX. Upon reaching

confluency, the serum-supplemented medium was replaced with the serum-free medium (CHO-S-SFM II, Gibco-BRL). The spent serum-free medium was exchanged with the fresh serum-free medium every 3 days over 12-day production period. The collected spent serum-free medium was centrifuged at 1,200 rpm for 10 min at 4°C. To insure high reproducible binding of the FLAG fusion protein, 10× TBS/Ca buffer was added to the centrifuged spent medium at a ratio of 1 to 9.

The rhAng2 and rhAng2$_{443}$ were purified at 4°C using anti-FLAG M1 affinity gel (1 mg/mL resin, Sigma). Briefly, the spent medium stored at 4°C (350 mL) was loaded onto the anti-FLAG M1 affinity column equilibriated with 1× TBS at a flow rate of ~ 0.5 mL/min. The column was washed three times with 12 mL aliquots of TBS/Ca. The bound FLAG fusion protein was eluted from the column with six 1 mL aliquots of 0.1 *M* glycine HCl (pH 3.5) into vials containing 25 μL of 1 *M* Tris (pH 8.0). The fractions containing rhAng2 or rhAng2$_{443}$ were concentrated using Centricon 50 (Amicon) at 4°C and stored at -70 °C until use.

SDS-PAGE and Western blotting The purified proteins were analyzed by 15% SDS-PAGE. Protein concentrations were determined by the BCA method. For Western blot analysis, 0.4μg of samples was mixed with reducing sample buffer or non-reducing sample buffer, boiled for 10 min, separated by SDS-PAGE, and electroblotted to ECL membranes. The ECL membranes were blocked in PBS buffer with 5% skim milk for 1 hour. Membranes were incubated with primary anti-Ang2 polyclonal antibody and the secondary antibody, a HRP-conjugated anti-mouse IgG.

RESULTS AND DISCUSSION

Expression of rhAng2 and rhAng2$_{443}$ in CHO cells For the high-level, stable expression of rhAg2 and rhAng2$_{443}$ in CHO cells, two expression vectors encoding hAng2 or Ang2443 were constructed as shown in Fig. 1. The FLAG epitope facilitates one-step purification of rhAng2 and rhAng2$_{443}$. The dhfr-deficient CHO cells were transfected with pAng2-dhfr or pAng2$_{443}$-dhfr vector using LipofectAMINE and were subjected to selection with G418 (500 μg/mL). On the basis of their relative specific angiopoietin productivity (q_{Ang}), 10 clones of Ang2 and Ang2$_{443}$ were selected for gene amplification and subjected to increasing levels of MTX (0.02, 0.08, and 0.32 μ*M*). Figure 2 shows q_{Ang} of 10 clones at each MTX level.

Purification of rhAng2 and rhAng2$_{443}$ Cells amplified at 0.08 μ*M* MTX (Ang2-3-0.08 for rhAng2 production and Ang2$_{443}$-4-0.08 for rhAng2$_{443}$ production) were seeded in T-175 cm^2. As described in materials and methods, secreted rhAng2 and rhAng2$_{443}$ then purified from the cultured medium using anti-FLAG-agarose affinity chromatography.

The total amounts of rhAng2 and rhAng2 in the cultured medium, which were determined by ELISA, were about 9.2 mg/L and 45.7 mg/L, respectively. Using anti-FLAG-agarose affinity chromatography, Approximately 3.4 mg of rhAng2 and 16.9 mg of rhAng2$_{443}$ could be recovered from 1 L of cultured medium. Thus, the purification yields of rhAng2 and rhAng2$_{443}$ were 36.83 and

37.0 %, respectively.

Activity of Ang2 and Ang2$_{443}$ Their biological activity *in vitro* of rhAng2 and rhAng2$_{443}$ was demonstrated by examining the effect of Ang2 and Ang2$_{443}$ on apoptosis in cultured human umbilical vein endothelial cells (HUVECs). Although Ang2 is known to be a naturally occurring antagonist of angiopoietin (Ang1) *in vivo*, the exact function of Ang2 itself is not known. Ang2 at high concentration, like Ang1, could be an apoptosis survival factor for endothelial cells through the activation of the Tie2 receptor.

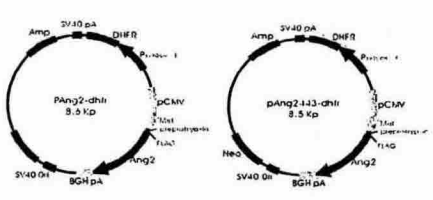

Figure 1. Map of pAng-dhfr and pAng2...-dhfr vector.

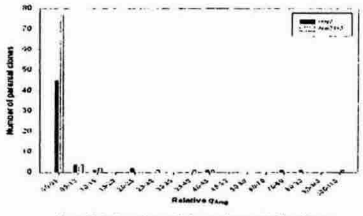

Figure 2. Analysis of parental clones with respect to the relative q_{Ang}

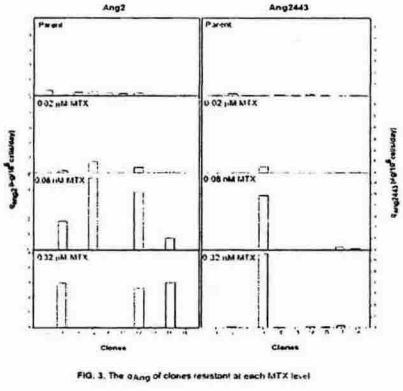

FIG. 3. The q_{Ang} of clones resistant at each MTX level

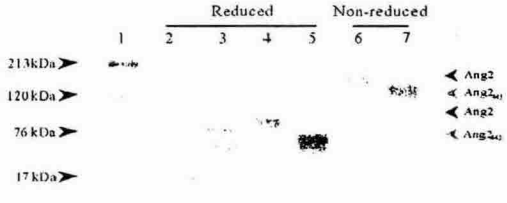

Figure 4. SDS-PAGE of purification of recombinant angiopoietin.

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