Efficient Production of sTNFRII-gAD Fusion Protein in Large Quantity by Use of the Modified CHO-S Cell Expression System

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Abstract

TNFα is one of the initial and important mediators to activate downstream signaling pathways by binding to trimerized TNFα receptors (TNFR), and thus is an ideal drug target for cancer therapy. Taking advantage of intrinsic homotrimORIZATION of the globular domain of adiponectin (gAD), we have developed a novel TNFα antagonist, the trimerized fusion protein named sTNFRII-gAD. However, our previously-used CHO expression system yielded less than 10 mg/L of sTNFRII-gAD. To produce large quantities of sTNFRII-gAD efficiently, we used a modified CHO-S cell expression system, which is based on a pHM3 vector with non-coding GC-rich DNA fragments for high-level gene expression. We obtained stable clones that produced 75 mg/L of sTNFRII-gAD in the 96-well plate, adapted the clones to 40 ml suspension serum-free batch culture, then optimized the culturing conditions to scale up the fed-batch culture in a 3 L shake-flask and finally in a 5 L AP30 bioreactor. We achieved a final yield of 52 mg/L of sTNFRII-gAD. The trimerized sTNFRII-gAD exhibited the higher affinity to TNFα with a dissociation constant (Kd) of 5.63 nM than the dimerized sTNFRII-Fc with a Kd of 13.4 nM, and further displayed the higher TNFα-neutralizing activity than sTNFRII-Fc (p < 0.05) in a L929 cytotoxicity assay. Therefore, the strategy employed in this study may provide an efficient avenue for large-scale production of other recombinant proteins by use of the modified CHO-S cell expression system.

Introduction

As an important cytokine, TNFα plays a pivotal part in many pathophysiological processes. In tissues, TNFα at low concentration shows beneficial effects, such as augmented of host defense mechanisms against infections, but at high concentration leads to inflammation and organ injury so as to cause diseases such as rheumatoid arthritis (RA), Crohn’s disease (CD), and psoriasis, etc. Moreover, acute release of large amounts of TNFα during sepsis may result in septic shock. Therefore, neutralization of TNFα has become an effective therapeutic strategy for these diseases. The TNFα antagonists approved by the US FDA for clinical use include bivalent sTNFRII-Fc (Etanercept) and two monoclonal anti-TNFα antibodies (Infliximab and Adalimumab) [1,2]. TNFα exerts its effects by binding, as a trimer, to either TNFRI or TNFRII. sTNFRII, the extracellular portion of TNFRII, is a natural TNFα antagonist [3]. However, monomeric sTNFRII has a lower affinity to TNFα, and also has a relative short half-life in circulation, thus resulting in lower therapeutic effects. It was reported that sTNFRII-Fc fusion protein could neutralize TNFα 50 to 1,000 times as much as monomeric sTNFRII due to the dimerization through the Fc moiety [4]. Therefore, enhancing the interaction between TNFα and sTNFRII has become one of the major approaches to develop novel TNFα antagonists.

Adiponectin (AD), a 30 kDa protein hormone consisting of a globular domain (gAD) and a collagenous domain, originates from adipose tissue and regulates numerous metabolic processes. The gAD is located at the carboxyl terminus and inclines to form a homotrimer. A collagenous domain within AD leads to spontaneous self-assembly into various oligomeric isoforms, including hexamers and high-molecular-weight multimers. The contribution of varying isoforms of AD to specific physiological processes
remains to be fully elucidated. Two membrane-spanning receptors for AD have been identified in various body tissues with differing distribution density. The major intracellular pathway activated by AD includes the phosphorylation of MAP-activated protein kinase, which is responsible for many of its metabolic regulatory, anti-inflammatory, vascular protective and anti-ischemic properties. Since its discovery in 1995 AD has garnered considerable attention for its role in diabetic and cardiovascular pathology. Clinical observations have demonstrated the association of hypoadiponectinemia in patients with obesity, cardiovascular disease and insulin resistance [5–9].

Utilizing the intrinsic trimerization property of gAD, we have developed a novel TNFα antagonist, the trimerized fusion protein named sTNFRII-gAD, which was composed of sTNFRII and gAD. We have shown that sTNFRII-gAD was superior to sTNFRII-Fc as a TNFα antagonist, highlighting the potential of sTNFRII-gAD for the treatment of excessive TNFα-associated diseases [10]. However, our previously reported sTNFRII-gAD expression system yielded less than 10 μg/L of sTNFRII-gAD. In an effort to produce large quantities of recombinant sTNFRII-gAD for further studies, here we reported the construction of a modified CHO-S expression system based on “GC-rich” vector pMH3 for high-level gene expression [11], and further developed a high density, full suspension serum-free fed-batch culture system for production of sTNFRII-gAD in large quantity with high yield.

Materials and Methods

Materials and Instruments

Restriction enzymes EcoRI, NotI, and T4 DNA ligase were purchased from Takara (Shiga, Japan). E.coli DH5α competent cells and pAAV2-sTNFRII-gAD vector were generated by our own laboratory. pMH3 vector, B001 serum-free basal medium, F001 feed medium, frustoconical-bottom shake bottles, and AP30 bioreactor were provided by Amprotein (Hangzhou, Zhejiang, China). pAAV2-sTNFRII-gAD vector were generated by our own laboratory. pMH3 vector, B001 serum-free basal medium, F001 feed medium, frustoconical-bottom shake bottles, and AP30 bioreactor were provided by Amprotein (Hangzhou, Zhejiang, China).

Cell Lines and Culture Conditions

The Chinese hamster ovary cell line (CHO-S) was kindly provided by AmProtein (Hangzhou, Zhejiang, China). CHO-S cells were grown in DMEM/F12 medium containing 10% FBS. For sTNFRII-gAD fusion protein expression, B001 serum-free basal medium and F001 feed medium were used. L929 cell was from ATCC (Manassas, VA, USA).

Plasmid Construction

The sTNFRII-gAD encoding gene was amplified by PCR using pAAV2-sTNFRII-gAD as template and under the following conditions: an initial denaturation of 2 min at 94°C was followed by 30 cycles of 15 s at 94°C, 30 s at 55°C, and 75 s at 68°C. Then a final elongation was performed for 10 min at 72°C. The forward and reverse primers were as follows: 5’-ACG GAA TTC GCC ACC ATG GCC CCC GTG GCC GT-3’ and 5’-AA AAG GAT ATA TGC GGC CCG TTA TCA TCA GTT GGT GTC GTG GTA CGG C-3’ (the underlined nucleotides of the primers denote the EcoRI and NotI sites, respectively). The PCR product was digested with EcoRI and NotI and then ligated to the expression vector pMH3, which was previously digested with these two enzymes. The expression plasmid pMH3-sTNFRII-gAD was purified from DH5α and the sequence of the resulting expression plasmid pMH3-sTNFRII-gAD was confirmed by DNA sequencing (Shanghai Bioengineering).

Acquisition of Stable sTNFRII-gAD-expressing Cell Clones with High Yield

The expression plasmid pMH3-sTNFRII-gAD was introduced into CHO-S cells by a gene pulser (Hercules, CA, Bio-rad). CHO-S cells were harvested by centrifugation (800 rpm, 3 min) and washed with PBS twice, then 5×10⁶ cells were gently suspended in 200 μl PBS. 25 μg pMH3-sTNFRII-gAD plasmid combined with 10 μl salmon sperm DNA was thoroughly mixed with 200 μl CHO-S cell suspension, which then was transferred into a chilled gene pulser cuvette. After 1 min on ice, electric shock with 160 V, 15 ms, followed by 1 min on ice immediately, then electric shock once again, the cell suspension was transferred into two 10 cm culture dishes with DMEM/F12 containing 10% FBS. After 24 h, the medium was replaced with selection medium containing 1.5 μg/ml G418 and 10% FBS. After about two weeks, neomycin resistant CHO-S/pMH3-sTNFRII-gAD cells were obtained. Following the above selection method, we obtained stable high expression clones in CHO-S in only one round G418 selection. The 2nd or 3rd clone selection was performed in order to get pure cell population. Subsequently, we acclimated the highest expressing clones to suspension culture in serum-free medium B001, then scaled up by using preliminarily optimized fed-batch cultures.

Production of sTNFRII-gAD

(i) Serum-free suspension batch culture. The hyperexpression cells were seeded at a concentration of 2×10⁶ cells/ml and cultured in frustoconical-bottom shake flasks with a working volume of 40 ml with serum-free medium B001 on a shaker at an agitation of 120 rpm at 37°C. On the first day of batch cultures, 8 g/L glucose was added. The cell growth, viability and sTNFRII-gAD expression (by dot blot) were evaluated daily. When the cell viability dropped to 60%, the batch cultures were terminated.

(ii) Serum-free suspension fed-batch culture. In order to avoid nutrient limitations in suspension batch culture, we first performed fed-batch cultures in 3 L frusto-conical-bottom shake flasks and then scaled up to 5 L AP30 bioreactor. 100 ml and 2 L
cultures of 2×10^6 cells/ml were inoculated to 3 L frosto-conical-bottom shake flasks and 5 L AP30 bioreactor with serum-free medium B001 on a shaker at 120 rpm and 55 rpm at 37°C, respectively. When the cell density reached to more than 4–6×10^6 cells/ml, feed medium F001 was added semi-continuously (e.g. daily or twice daily) to keep the glucose concentration at 2 g/L; meanwhile, the temperature was gradually reduced to 34°C. The culture in AP30 bioreactor was controlled by an on-line computer: pH 7.00 ±0.1, dissolved oxygen (DO) of 55% air saturation, and agitation at 55 rpm. The samples were analyzed daily to determine the cell viability, density, and metabolites. They were also frozen for yield analysis later via dot blot. When the cell viability dropped to 60%, the fed-batch cultures were terminated and the supernatants were harvested, filtered with a 0.22 μm cellulose acetate filter, and stored at −20°C for future use.

Figure 2. Dot blot analysis of sTNFRII-gAD protein in 96-well plates at 24 h with monoclonal antibody against TNFRII. Lane 1: 100, 75, 50, 25, 12.5 and 6.25 μg/ml of sTNFRII-Fc; Lane 2, 3, 4: 1/1, 1/3 and 1/6 dilutions of the supernatants from the 1st, 2nd and 3rd hyper-expression clones.

doi:10.1371/journal.pone.0111229.g002

Figure 3. Western blot analysis of sTNFRII-gAD in culture supernatants using monoclonal antibody against adiponectin (A) and sTNFII (B). Lane 1, molecular weight markers (kDa); Lane 2, reducing conditions; Lane 3, nonreducing conditions.

doi:10.1371/journal.pone.0111229.g003
USA) was used as the primary antibody at a concentration of 1:3,000. The alkaline phosphatase substrate was BCIP/NBT (Calbiochem Corporation). The peroxidase substrate was 3-amin-9-ethylcarbazole (ECL). Western blotting detecting reagent, Amersham).

Affinity Measurement for Binding of sTNFRII-gAD to TNFα
Surface plasmon resonance (SPR) measurements were performed using a Biacore 3000 instrument (Biacore International AB, Uppsala, Sweden). TNFα was immobilized on a CM5 sensor chip at concentrations of 50 μg/ml in 10 mM sodium acetate, pH 4.0, using N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) -carbodiimide hydrochloride (NHS/EDC) at a flow rate of 10 μl/min. Binding of the purified trimerized sTNFRII-gAD fusion protein and commercial sTNFRII-Fc to the immobilized TNFα was measured by using serial dilutions of sTNFRII-gAD and sTNFRII-Fc from 130 to 4.0625 nM at a flow rate of 30 μl/min, respectively. Then they were eluted by 10 mM sodium hydroxide at 30 μl/min for 30 s, followed by PBS-T (PBS+0.005% Tween) buffer stabilization for 3 min. The dissociation constant (Kd) was derived from a linear regression of steady state 1/Response versus 1/C double reciprocal plots as well as by fitting of binding kinetics using a first-order Langmuir model.

sTNFRII-gAD Bioassays
The biological activity of the sTNFRII-gAD fusion protein was assessed by a TNFα-induced L929 cytotoxicity assay. Briefly, L929 cells were plated in a 96-well plate (1.5×10^4 cells/well) and were incubated for four hours. The media was removed, and 100 μl of a 5-mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) solution was added to each well and incubated for 4 hours. The media was removed, and 100 μl of dimethylsulfoxide was added to each well to dissolve the formazan dye for 30 minutes at 37°C. Absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm with a microplate reader. Commercial sTNFRII-Fc was used for comparison.

Results
Construction of the sTNFRII-gAD Expression Plasmid
The CHO cell line was one of the most important systems for expression of foreign gene, but the expression level was low.

Table 1. Comparison of the Growth and sTNFRII-gAD Fusion Protein Production of the Engineered Cells in Suspension Culture.

| Culture time (d) | 40 ml batch | 3 L fed-batch | 5 L fed-batch |
|------------------|-------------|---------------|--------------|
| Max conc (10^6 cells/ml) | 8.00 | 17.00 | 18.00 |
| Supernatants collected (L) | 6.00 | 9.20 | 8.90 |
| Protein concentration (mg/L)^a | 0.04 | 1.80 | 4.60 |
| Protein purity (%)^b | 13.00 | 40.00 | 52.00 |

^aEstimated by BCA protein assay after anion exchange chromatography.
^bDetermined by densitometry of gel (data not shown).

doi:10.1371/journal.pone.0111229.t001
Productivity of cell culture titer can be increased through the modulation of transcriptional activity via expression vector engineering by modulating the co-expression of product and selection marker genes, the stringency of the selection marker, the DNA regulatory elements carried on the vector, and targeting its integration site on the host cell genome [13]. To increase the protein yield, we chose pMH3 plasmid with GC-rich non-coding DNA fragments, which were crucial for chromatin openness [11]. The gene structure of pMH3-sTNFRII-gAD expression plasmid used for sTNFRII-gAD expression is shown in Fig. 1 and contains three GC-rich non-coding DNA fragments at the 5’ and 3’ flanking regions of sTNFRII-gAD gene and actin promoter.

Selection of Stable sTNFRII-gAD-expressing Cell Clones with High Yield

pMH3-sTNFRII-gAD plasmid was transfected into CHO-S cells via electroporation. The expression level of sTNFRII-gAD in 96-well plates reached up to 75 μg/ml after only one round of G418 selection in three weeks, as assessed by dot blotting analysis. Two rounds of clonal purification were undertaken subsequently, resulting in no dramatic change in expression level (Fig. 2).

Furthermore, the supernatants from the selected hyper-expression clones were analyzed by western blot with monoclonal antibodies against sTNFRII or adiponectin. Three antibody-specific bands with approximate apparent molecular weights of 50, 150, 250 kDa were identified in non-reducing conditions, which represented monomer, trimer, and multimer form of sTNFRII-gAD, respectively (Fig. 3A, 3B). In reducing conditions, there
existed one specific bands with approximate apparent molecular weights of 50 kDa, the band was recognized by anti-adiponectin (Fig. 3A), while not by anti-TNFRII (Fig. 3B). This may due to the fact that anti-TNFRII could only recognize spatial epitope of sTNFRII (not linear epitope), while anti-adiponectin could identify linear epitope of gAD [14].

Production of sTNFRII-gAD Fusion Protein

The hyper-expression clones were adapted to high density, serum-free suspension culture by use of chemically-defined medium that was capable of producing sTNFRII-gAD. The culturing time of suspension batch was much shorter than that of fed batch. The yields of sTNFRII-gAD in the fed batch were about 3–4 times more than that in suspension batches (Table 1). High density, high viability, and high yields in fed-batch cultures may be mainly due to the preliminarily optimized feeding strategy, such as high inoculation density of 2×10^6 cells/ml, later time to feed as the cell density expanding to 6–8×10^6 cells/ml, maintenance of lower glucose level of 2 g/L (11 mM), and decrease in temperature to 34°C at the production stage. We found that kept glucose level at 2 g/L resulted in low lactate concentration, which may prevent cell death (Fig. 4). We also found that when fed batch scaled up to 5 L in an AP30 bioreactor, cells still remained high density, high viability, and high yields, indicating that the performances achieved on the small scales (3 L) could be reliably expanded to the large scale.

Purification of sTNFRII-gAD Fusion Protein

The harvested supernatants were concentrated by 20-fold through a cascade TFF system before sTNFRII-gAD fusion protein purification. The concentrates were subsequently loaded onto an anion exchange column. The elution revealed one major protein peak (Fig. 5A). The eluted sTNFRII-gAD fusion protein showed three major bands on SDS-PAGE gel with approximate apparent molecular weights of 50, 150, 250 kDa, which represented monomer, trimer, and multimer forms, respectively.

Figure 6. SDS-PAGE and western blot analyses of the purified sTNFRII-gAD. (A) SDS-PAGE analysis of sTNFRII-gAD purified by anion exchange chromatography. Lane 1: molecular weight markers (kDa); lanes 2, 3: The loaded protein samples were about 5.0 µg each loading in non-reducing and reducing conditions, respectively. (B) SDS-PAGE analysis of sTNFRII-gAD separated by HiLoad 16/60 Superdex 200 chromatography. Lane 1, protein molecular weight markers; Lane 2, multimeric sTNFRII-gAD (peak 1); Lane 3, trimeric sTNFRII-gAD (peak 2); Lane 4, monomeric sTNFRII-gAD (peak 3). The loaded protein samples were about 2.0, 5.0, and 3.0 µg, respectively. (C) Western blot analysis of purified trimeric sTNFRII-gAD under non-reducing/reducing conditions. Lane 1, molecular weight markers; Lanes 2 and 3: The loaded protein samples were about 5.0 µg and 3.0 µg in non-reducing and reducing conditions, respectively.

doi:10.1371/journal.pone.0111229.g006

Figure 7. Ultracentrifuge analysis of the trimerized sTNFRII-gAD. It gave a molecular weight of 165 kDa.

doi:10.1371/journal.pone.0111229.g007
The eluted protein was further purified to homogeneity using a size exclusion column, giving rise to three major protein peaks (Fig. 5B). SDS-PAGE analysis revealed that the peaks 1, 2, 3 were multimers, trimers, and monomers respectively (Fig. 6B). The identity of the purified sTNFRII-gAD was further confirmed by western blot assay (Fig. 6C), as well as by N-terminal sequencing (L-P-A-Q-V). Furthermore, ultracentrifuge analysis of the trimerized sTNFRII-gAD indicated a molecular weight of 165 kDa (Fig. 7), consistent with the SDS-PAGE result (150 kDa).

Binding Affinity of sTNFRII-gAD to TNFα

Direct binding of recombinant sTNFRII-gAD/sTNFRII-Fc to TNFα was measured through Biacore technology. By analysis of the sensorgram curve, the apparent dissociation constant (Kd) of the purified sTNFRII-gAD was determined.

Figure 8. Biacore sensorgram of sTNFRII-gAD/sTNFRII-Fc binding to TNFα immobilized on a CM5 chip. Binding measurements were performed using serial dilutions of sTNFRII-gAD and sTNFRII-Fc. The concentrations of sTNFRII-gAD were 130, 65, 32.5, 16.25, 8.125, and 4.0625 nM (A), while the concentrations of sTNFRII-Fc were 520, 260, 130, 65, 32.5, 16.25, and 8.125 nM (B).

doi:10.1371/journal.pone.0111229.g008

Figure 9. Biological activity of purified sTNFRII-gAD. An asterisk symbol (*) indicated a statistically significant difference (p<0.05) between sTNFRII-Fc and sTNFRII-gAD. All assays were performed in triplicate, with error bars representing the standard error of the mean of the samples.

doi:10.1371/journal.pone.0111229.g009
the purified trimered sTNFR-II-gAD and a commercial sTNFR-II-Fc for TNFα were 5.63 nM and 13.4 nM, respectively (Fig. 9), with on kinetic constants of 1.03×10−5 1/ms and 4.32×10−5 1/ms, and off kinetic constants of 2.43×10−3 1/s and 2.43×10−3 1/s for sTNFR-II-gAD and sTNFR-II-Fc, respectively.

Biological Activity of sTNFR-II-gAD Fusion Protein

Bioassays were conducted to measure the biological activity of the purified recombinant sTNFR-II-gAD fusion protein to antagonize TNFα-induced 1,299 cytotoxicity, and compare its activity with sTNFR-II-Fc. The dose-response curves showed that both sTNFR-II-gAD and sTNFR-II-Fc neutralized TNFα effectively in a dose-dependent manner. Neutralizing activity of sTNFR-II-gAD was significantly higher than that of sTNFR-II-Fc (p<0.05) (Fig. 9), which is consistent with our previous observation showing that sTNFR-II-gAD was able to attenuate D-galactosamine/LPS-induced acute liver injury resulted from excessive TNFα more efficaciously than sTNFR-II-Fc [10].

Discussion

A novel TNFα antagonist, sTNFR-II-gAD, was first successfully expressed in a mammalian expression system in our lab, although with low yields [15]. Subsequently, we modified the coding sequence of the sTNFR-II-gAD gene in order to increase the expression level without altering the amino acid sequence, followed by using a dihydrofolate reductase gene for gene amplification to increase protein expression in CHO/dhfr cells. However, the yield reached to only 8 mg/L in bioreactors [15].

In this report, high level expression of recombinant sTNFR-II-gAD fusion protein was achieved by use of a “GC-rich” expression vector. An alternative non-coding GC-rich DNA fragment was proposed to be a novel ubiquitous chromatin opening elements (UCOE), since flanking the gene of interest with the GC-rich fragment augmented recombinant protein expression [11]. UCOE was reported to prevent gene silencing consistently and resulted in stable and high expression of the gene of interest regardless of the chromosomal integration site [16]. Use of the pMH3-sTNFR-II-gAD construct resulted in 75 μg/ml of sTNFR-II-gAD in a 96-well plate after only one round of G418 selection. More importantly, the expression levels in scale-up cultures were relatively stable, with a final yield of 32 mg/L (Table 1). This represents a 5 to 6-fold improvement in sTNFR-II-gAD production as compared with our previous expression system, the most commonly used CHO/DHFR expression system. The system suffered from requiring a low-glucose environment produced less lactate and improved protein production [20–22]. Additionally, lowering temperature is an effective method to control cell proliferation [23]. Several studies showed that the temperature shift from 37°C to 30–35°C at 48 hours post inoculation could retain cells in G1 phase longer, and therefore delayed the onset of apoptosis and enhanced cell growth and cell viability in late period of the culture. The determination of a key substrate, e.g. glucose, would be ideal for feeding control. Previous studies indicated that the by-products, such as lactate, could decrease by maintaining low glucose concentrations through frequent or continuous feeding [19]. Due to metabolic shift, adapting cells to a low-glucose environment produced less lactate and improved protein production [20–22].

In conclusion, we constructed a modified CHO-S expression system for sTNFR-II-gAD fusion protein on basis of “GC-rich” vector pMH3 for high-level gene expression, and further developed a high density, full suspension serum-free fed-batch culture system for production of sTNFR-II-gAD in large quantity with high yield. Thus, the strategy employed in this study may provide an efficient avenue for large-scale production of other recombinant proteins by use of the modified CHO-S cell expression system.

Acknowledgments

We would like to thank Dr. Muzhou Hui at AmProtein China, Hangzhou for his technical support.

Author Contributions

Conceived and designed the experiments: QC YL JG. Performed the experiments: QC AZ YY LM ZJ HZ. Analyzed the data: QC AZ JG. Contributed reagents/materials/analysis tools: YY LM ZJ HZ. Contributed to the writing of the manuscript: QC KS JG.

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