Inhibition of the ubiquitin ligase activity improves the production of biologically active fusion protein HSA-HGF in Chinese hamster ovary cells

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ABSTRACT
Hepatocyte growth factor (HGF) is a potent multi-functional protein that stimulates proliferation, survival, motility, scattering and differentiation during growth and development, and has been considered to be a potential therapeutic agent for the treatment of a number of intractable diseases. The aim of this study was to enhance the expression of recombinant fusion protein HSA-HGF (R494E) in CHO cells by inhibiting the intracellular ubiquitin ligase activity. The high stable expression sub-clones with different signal peptides were selected by western blot (WB) analysis and used for suspension culture. We found that the expression of fusion protein HSA-HGF (R494E) on day 3 achieved 50 mg/L during the 8 day culture process, a large number of fusion proteins were intracellular degraded by ubiquitination pathway during day 4 to day 8. Furthermore, ubiquitin ligase inhibitor, thalidomide, was added in culture process, and resulted in efficient and stable secretion of HSA-HGF (R494E) in CHO cells. According to biological activity assays, HSA-HGF (R494E) possessed various biological activities similar to native HGF. In conclusion, inhibition of intracellular ubiquitin ligase activity was successfully improve the expression of biologically active fusion protein HSA-HGF (R494E) in CHO cells. Our data may be beneficial to enhance the production of other therapeutic proteins in fed-batch culture.

Introduction
Hepatocyte growth factor (HGF) was originally identified as the most potent mitogen for primary hepatocytes. In vivo, HGF is a pleiotropic factor that stimulates proliferation, survival, motility, scattering and differentiation during growth and development. Knock out mice have demonstrated that HGF is necessary for normal embryogenesis. In clinically treatment, HGF exhibits pleiotropic effects and contributes to mitogenic, anti-inflammatory, anti-apoptotic and anti-fibrogenetic activities. Depending on many independent studies, HGF has shown potential as a therapeutic agent for the treatment of a number of intractable diseases.

During the past decade, extensive studies to address the therapeutic effects of HGF have provided enough significance to develop agonists of HGF as biological drugs in regeneration-based therapies, drug development using HGF remains a challenge. However, short half-life of HGF in the blood circulation is only 3–5 min, which makes it hard to maintain the constantly level in patients. In previous reports, the short half-lifes of recombinant pharmaceutical proteins were improved by fusion technology. Several biological drugs improved the therapeutic activity such as human growth hormone (GH) fused with HSA were used effectively to create long-lasting therapeutic drug candidates. As a fusion partner, human serum albumin (HSA) was commonly used to prolong the short half-life due to its several advantages, such as abundant in serum, long half-life (~19 days) and low immunogenicity.

Our group has worked for years to make recombinant human HGF available as a biological drug.
Human HGF is secreted as an inactive 728 amino acid (aa) single chain precursor protein. It is cleaved after the fourth Kringle domain by a serine protease to form bioactive disulfide-linked heterodimers with a 60 kDa $\alpha$-subunit (the 4 Kringle domain) and 30 kDa $\beta$-subunit. In our previous work, we found that the cleavage site of HGF was mutated by replacing the Arg 494 with Glu and the obtained HGF (R494E) possessed similar biological activity to the native HGF. Subsequently, we created a fusion protein using human serum albumin (HSA) and biological active HGF (R494E) to prolong half-life of HGF (R494E). The fusion protein HSA-HGF (R494E) may be the suitable protein for the drug reagent development.

In present study, albumin fusion technology was applied to improve the short half-life of HGF by constructing a fusion protein expressing CHO cell line.

We established a CHO cell line overexpressing fusion protein HSA-HGF (R494E) to be used for large scale production. The secretion of HSA-HGF (R494E) accumulated in the medium during the 8 day culture process reached 50 mg/L, more than HGF (R494E) expression level of 12 mg/L. However, the expression of fusion protein HSA-HGF (R494E) on day 3 achieved 50 mg/L, a large number of fusion proteins were intracellular degraded by ubiquitination pathway during day 4 to day 8. The synthesis of protein is easily affected by cell stresses, which leads to misfolding of protein and results in dysfunction of the respective protein. The ubiquitin proteasome pathway is responsible for the degradation of short lived and dysfunctional proteins in the eukaryotic cell. The aim of this study was to enhance the expression of HSA-HGF (R494E) by inhibition of the ubiquitin ligase activity for efficient and long-time production. The biological activity of HSA-HGF (R494E) were investigated and compared with native HGF. The results of this study showed that HSA-HGF (R494E) possessed similar biological activity to the native HGF. The inhibition of intracellular ubiquitin ligase activity enhanced the expression of fusion protein HSA-HGF (R494E) in CHO. It will be beneficial to other similar therapeutic proteins production in CHO cells.

Results

Establishment of HSA-HGF(R494E) expression cell line

The survival cells under G418 was transferred to 96-well microplates and the concentration of HSA-HGF (R494E) supernatants were analyzed by dot blot (Fig. 1A). Subsequently, the high sub-clones from dot blot result was transferred to 24-well microplate for further WB analysis (Fig. 1B). After screening by dot blot and WB, the high fusion expression sub-clone was used for further suspension culture. In fed-batch suspension culture with serum-free culture medium

![Figure 1. Selection of stable HSA-HGF (R494E)-expressing cell clones by dot blot (A) and WB (B) analysis. Standard: various HSA concentration of 20, 10, 5 and 2.5 mg/L; 1–7 were selected cell clones.](image_url)
(SFM), viable cell density increased from day 1 and peaked on day 3 with \(8 \times 10^6\) cells/mL and the cell viability exceeded 95% during the whole culture process (Fig. 2A). Meanwhile, HSA-HGF(R494E) concentration during suspension culture achieved the highest on day 3 with 50 mg/L (Fig. 2B).

**Purification of fusion protein HSA-HGF(R494E)**

The supertanant of CHO cells for fusion protein expression was obtained and purified by a 2-step strategies, including SP Sepharose Fast Flow column and HiTrap Heparin HP column. Different collected fractions were analyzed by SDS-PAGE after purification, and results indicated that we successfully obtained the target fusion protein with molecular weight (Mw) of 140 kDa (Fig. 3). After detecting total protein and HSA-HGF(R494E) concentrations, the yield and purity of HSA-HGF(R494E) were 50% and 90%, respectively (Table 2).

**Scattering activity of MDCK cells by HSA-HGF(R494E)**

HGF induces cell scattering, a phenomena characterized by previous reports and the cell-cell connections were mediated by cadherin which allowed cells to migrate.\(^{21}\) The cell scattering assay is the classical method to identify biological activity of HGF.\(^{22}\) The biological activity of purified HSA-HGF (R494E) was determined using a scattering activity assay with Madin-Darby Canine Kidney (MDCK) cells. The scattering activity assay were carried out by negative control without proteins (Fig. 4A), positive control with 0.25 nM native HGF protein (purchased from R&D) (Fig. 4B), 8.5 nM HSA expression from yeast (Fig. 4B), 8.5 nM purified HSA-HGF (R494E) (Fig. 4C), 0.25 nM native HGF with specific blocking antibody to HGF (Fig. 4D), 8.5 nM purified HSA-HGF (R494E) with specific blocking antibody to HGF (Fig. 4E). Our previously work proved that the cleavage site mutated HGF by replacing the Arg 94 with Glu possessed similar biological activity to the native HGF (0.25 nM) at the high concentration (1.25 nM). In addition, the fusion protein HSA-HGF (R494E) in this study induced cell scattering similar to native

![Figure 2. Cell growth and HSA-HGF (R494E) expression of in serum-free suspension fed-batch culture. (A) Viable cell density and cell viability. (B) SDS–PAGE analysis of each day supernatants for the 8 d fed-batch culture.](image1)

![Figure 3. Purification of HSA-HGF (R494E). SDS–PAGE analysis of HSA-HGF (R494E) purified process, L: loading medium for SP column; F: flow through; A1-A4: fractions eluted by 20 mM Tris buffer (pH 8.5); A5-A8: fractions eluted by 20 mM Tris buffer (pH 8.5), 0.4 M NaCl; A9: fraction eluted by heparin affinity.](image2)

**Table 1.** Primer sequences for PCR.

| Primers    | Sequences                                                                 |
|------------|----------------------------------------------------------------------------|
| Forward    | Gcgaattccacatgagacagacacactctgtaggtactgctgctctgggttccaggttccactggtgatgcacacaagagtgaggttgctcatcgatttaaag |
| Reverse    | agcggccgcctatgactgtggtaccttatatgttaaaataa                                    |

**Table 2.** Summary of HSA-HGF (R494E) in each step from 3 L suspension fed-batch culture.

| Steps                | Total protein (mg) | HSA-HGF (R494E) (mg) | Yield (%) | Purity (%) |
|----------------------|--------------------|----------------------|-----------|------------|
| Crude supertanant    | 250                | 50                   | 100       | 20         |
| Ion exchange column  | 40                 | 30                   | 60        | 75         |
| Affinity column      | 28                 | 25                   | 50        | 90         |

Estimated by BCA protein assay, ELISA and coomassie blue-stained SDS–PAGE gel.
HGF and the scattering activity was limited by rabbit polyclonal anti-HGF. These results indicated that purified CHO expressed HSA-HGF (R494E) had functionally activity at the high molar concentration. HSA maybe influenced the binding of receptor c-MET but could not affect signal transduction.

**HSA-HGF (R494E) stimulated signal transduction in L02 cells**

Effect of HSA-HGF (R494E) on tyrosine kinase activity of receptor c-MET in hepatocyte L02 cells was detected by commercial kits. The results showed that HSA-HGF (R494E) stimulated signal transduction in L02 cells (Fig. 5A) and increased the phosphorylation of AKT (Fig. 5B). Therefore, these results indicated that HSA-HGF (R494E) had several effects for various c-MET receptor positive cells. Besides, HSA-HGF (R494E) stimulated signal transduction steady and maintained over 8 h corresponding to native HGF for less than 1 h in L02 cells (supplement 1).

**Enhanced HSA-HGF (R494E) by adding thalidomide**

In the 8-day fed-batch culture process, secreted HSA-HGF (R494E) in medium influenced intracellular metabolic pathway to CHO. The expression of fusion protein HSA-HGF (R494E) on day 3 achieved 50 mg/L, and almost no fusion protein accumulation during day 4 to day 8 (Fig. 6B). According to transcription analysis, hsa-hgf mRNA level sharply reduced from day 2 to day 4 and then slowly increased after day 4 to the end of culture, the transcription level in day 4 was about 25 percent of day 1 (Fig. 6C). Most of all, there are almost no detectable intracellular fusion proteins during day 4 to day 8 despite of massive intracellular HSA-HGF (R494E) were detected during day 1 to day 3 (Fig. 6A). There is no accumulation of extracellular proteins without intracellular proteins.

Thalidomide, a kind of inhibitor of ubiquitin ligase, is capable of inhibition of intracellular degradation pathway. We supplement with 20 mg/L thalidomide at interval 2 d in the 8-day fed-batch culture process for HSA-HGF (R494E). The results showed that transcription levels increased to normal levels (Fig. 6C) and massive intracellular fusion proteins were detected in the whole culture process (Fig. 6A). Most important of all, HSA-HGF (R494E) accumulated

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**Figure 4.** HSA-HGF (R494E)-mediated MDCK cells scattering assay. (A) Control without any treatment; (B) 8.5 nM HSA; (C) 20 ng/ml (0.25 nM) native human HGF protein; (D) 800 ng/ml (8.5 nM) purified HSA-HGF (R494E); (E) Human HGF plus blocking antibody; (F) HSA-HGF (R494E) plus blocking antibody.
clearly in medium day by day (Fig. 6B). We conferred that fusion proteins were degraded by ubiquitination pathway during day 4 to day 8.

Discussion

Albumin with Mw of 67 kDa is the most abundant protein in the blood and responsible for 80% of the osmotic pressure of plasma. In previous studies, the short half-life of recombinant pharmaceutical proteins were improved by fusion technology. Albumin fusion technology notably prolongs the half-life of recombinant proteins in vivo by the fusion with human serum albumin (HSA). Besides, the enhanced pharmacodynamics of fusion proteins were related to its extended circulating half-life, low clearance, and stability in circulation. Compared to their native proteins, the lower binding affinity and biological potency (about 10 percent or less) in vitro assay had shown in most HSA fusion product. It was surmised that the increase in molecular size after small parent molecules fused with albumin possibly reduced the numbers of receptors occupied, HSA may influenced the binding of receptor but could not affect signal transduction.

HGF has shown potential as a therapeutic agent for the treatment of multiple liver diseases, because HGF inhibits development of liver fibrosis as well as promoting liver regeneration. Drug development using HGF was questionable because of its short half-life period of only 3–5 min, which was extremely unstable in the blood circulation. In recent experiments, HGF was encapsulated with ultrasound-targeted microbubble or liposomes to preserve its structure and integrity, allowing a prolonged release profile that was necessary to promote the treatment effect. However, these encapsulated particles are not suitable to pharmacetic preparation. Therefore, HSA-HGF (R494E) may be the suitable protein for long-lasting therapeutic drug candidates due to the metabolic stability of HSA and the reduction of renal clearance of fusion proteins.

In this study, high concentration of HSA-HGF (R494E) in culture medium affected the further expression of the fusion protein during day 4 to day 8 in suspension culture. The transcription level sharply reduced from day 2 to day 4, and slowly increased after day 4 to the end of culture. Besides, extremely low amount of intracellular fusion proteins during day 4 to day 8 was detected. It seemed that the synthesis of HSA-HGF (R494E) after day 4 was inhibited by trancription level. In the study of our grout, the fusion protein with HSA during suspension culture had no negative effect on growth and cell cycle of CHO cells, and had no effect on expression of target protein (data was not show). Therefore, the low expression of HSA-HGF (R494E) may be caused by the decreasing of transcription level and intracellular fusion protein degradation. In this study, we focused on the the intracellular degradation of fusion protein to enhanced the recombinant protein expression. A ubiquitin ligase inhibitor, thalidomide, was added during suspension culture. The addition of thalidomide resulted in the enhanced transcription levels to normal levels, and intracellular HSA-HGF (R494E) accumulated after day 4. It seemed that the low transcription level and degradation of intracellular HSA-HGF (R494E) were caused by ubiquitin proteasome pathway. Moreover,
the inhibition of intracellular ubiquitin ligase activity improve the expression of biologically active fusion protein HSA-HGF (R494E) in CHO cells. Therefore, the development of fusion protein HSA-HGF (R494E) is helpful for its clinical treatment and will be beneficial to other similar therapeutic proteins development.

**Materials and methods**

**Plasmid, cell line and culture medium**

Plasmid pMH3 and Cell line CHO K1 were all purchased from Amprotein Co. (Hangzhou, China). Culture medium for adherent cells was DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). CHO cells were cultured at 37°C, 5% CO2. Serum-free medium (SFM) B001 and fed medium F001 used for suspension culture were purchased from Amprotein Co. (Hangzhou, China).

**Construction of expression plasmid**

The purchased plasmid pMH3 contained high GC region of chicken β-actin on both sides of multiple clone site. The structurally rigid GC-rich segments support the opening of chromatin, thus exposing the promoter/enhancer regions to transcriptional factors and DNA binding proteins, resulting in high transcriptional levels of target genes. The encoding genes of HGF (R494E) were preserved in our lab and used.

![Figure 6](image-url)
as template for fusion PCR. The primers used in this study were listed in Table 1. The PCR products and plasmid were digested by EcoR I and Not I and then ligated to obtain the expression plasmid, which was identified by sequencing (Shanghai Sangon).

**Establishment of fusion protein expression cell line**

The constructed plasmids were transfected to CHO K1 cells by electroporation with condition of 400 V, 40 μs. The electroporation reaction mixture contained: 3 × 10^6 cells, 20 μg plasmid, 5 μg salmon sperm DNA. Transfected cells were cultured in DMEM/F12 with 10 % FBS for 2 d and then added 1.8 mg/L G418 for selection. Then the cell clones were picked and cultured in 96-well plates for 7 d.

The high expression sub-clones were evaluated at 24 h after replacing the medium of serum-free medium B001. For the second selection cycle, 100 first selected cycle cells were seeded in a 9 cm dish and selected again similar to the above mentioned.

The final high expression sub-clones were passaged and selected gradually from 96 well plate to 24 well plate and finally to 6 well plate. The high expression sub-clones were detected by dot blot from 96, 24 well plates and western blot from 6 well plates, and then used for suspension culture.

**Suspension culture and purification**

CHO cells purchased from Amprotein Co. were suspend-adapted cells and thus high expression sub-clone of fusion protein was used for suspension culture. Cells were inoculated to 3 L cone bottom shake flask (working volume 1.2 L) with a final density of 2.0 × 10^6 cells/mL and cultured at 37°C, 100 rpm for 2 d. Then culture temperature reduced to 34°C and F001 was added to cell culture to maintain a glucose concentration of 3.0 mg/L. Viable cell density was analyzed by the trypan blue dye method and HGF concentration were detected by using commercial Elisa kit from R&D (accession #:DHG00).

The culture supernatants of CHO cells were centrifuged by 2000 rpm for 10 min and filtered by 0.45 μm filters for further purifications. After equilibrating by 20 mM

30 sodium phosphate buffer (pH 6.0), SP Sepharose Fast Flow column (GE) was loaded with supernatant (final pH 6.0). Proteins in column was first washed by 20 mM Tris buffer (pH 8.5) and then fusion protein was eluted by 20 mM Tris buffer (pH 8.5) with 0.4 M NaCl. The collected fusion protein was diluted by 0.3 volumes of distilled water and then loaded to HiTrap Heparin HP column (GE). The column was equilibrated by 10 mM Tris (pH 7.5) with 0.3 M NaCl and fusion protein was eluted by a linear gradient of 0.3–2 M NaCl solutions. The purification process in this study was similar to previously work. All collected fraction during the protein purification was analyzed by sulfate-polyacrylamide gel electrophoresi (SDS-PAGE).

**Biological activity assays**

The biological activity of HSA-HGF (R494E) were compared with native HGF purchased from R&D (accession #: 294-HGN). Scattering activity was evaluated using an MDCK (Madin–Darby Canine Kidney cells) scattering assay as previously described. The specific blocking antibody to HGF was provided by Boliang Cao, the co-worker of the author of Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models. The activated receptor protein tyrosine kinase of L02 cells were detected by commercial tyrosine kinase assay kit (TaKaRa; Dalian, China). The phosphorylation downstream signaling molecules P-AKT detected by western Blot.

**Western blot and dot blot**

For Western blot, fusion proteins HSA-HGF (R494E) in SDS-PAGE were transferred to PVDF membranes and blocked by 5 % skim milk. The membrane was incubated with mouse monoclonal anti-human HGF (R&D, USA) at 4°C overnight and secondary goat anti-mouse antibody conjugated with HRP for 1 h. For dot blot, 5 μl samples were dripped onto the PVDF membranes and blocked by 5 % skim milk for 1 h at room temperature. Then membrane was incubated with rabbit monoclonal anti-human HSA antibody (abcam, UK) for 1 h at room temperature and secondary goat anti-rabbit antibody conjugated with HRP for 1 h.

**RNA and cDNA preparation, and quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from collected CHO cells using a Trizol RNA purification kit (Sangon; Shanghai, China) and reverse transcription was carried out.
by first strand cDNA synthesis kit (TaKaRa; Dalian, China) to obtain cDNA. Transcriptional levels of hgf were analyzed by qRT-PCR using SYBR green (TaKaRa, Dalian, China) according to the manufacturer’s instructions. Product specificity was confirmed by melting curve analysis. In all cases, gene expression levels were normalized against β-actin.

**Disclosure of potential conflicts of interest**
No potential conflicts of interest were disclosed.

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