Cloning, Expression and Purification of a novel anti-angiogenic factor-Tumstatin

Research Article

Chongbi Li1,* , Liming Yang2, Hongli Jia3
1The Center of Biopharmaceutical Research and Development of Zhaoqing University, 526061, China (PR)
2Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China (PR)
3The Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing 100071, China (PR)

*Correspondence: Chongbi Li, Biochemistry and Molecular Biology, The Center of Biopharmaceutical Research and Development of Zhaoqing University, 526061, China (PR); Tel: (86-0758)2752578; E-mail: lchongbi@yahoo.com

Key words: tumstatin, cloning and expression; IMAC

Abbreviations: immunoblot, metal-chelating affinity chromatography, (IMAC); Luria-Bertani, (LB); noncollagenous 1, (NC1); reverse transcription, (RT); vascular endothelial growth factor, (VEGF)

This study has laid a foundation for manufacturing anti-tumor based on Tumstatin.

Received: 29 May 2006; Revised: 12 June and 13 July 2006
Accepted: 17 August 2006; electronically published: September 2006

Summary

Tumor progression may be controlled by various fragments derived from noncollagenous 1 (NC1) C-terminal domains of type IV collagen. Tumstatin peptide is an angiogenesis inhibitor derived from type IV collagen and inhibits in vivo neovascularization induced by vascular endothelial growth factor (VEGF). Here, we firstly showed the expression, cloning and purification of tumstatin from Chinese abortus kidney tissue by RT-PCR, and the construction of pET-His expressive plasmid in prokaryotic cells. Also its’ activity was examined by mouse antisera against native Tumstatin. The results indicated E.coli BL21(DE3)plysS/pET-His-tumstatin was induced 3 h by 0.2 mmol/L IPTG at 30°C, and got a high-level expression of 37.9%. The Tumstatin protein was one-step purified by immobilized metal-chelating affinity chromatography (IMAC) and its purity was above 95%. Western blot identified it’s right.

I. Introduction

Tumstatin is a ramification of basement membrane proteins in human body (28Kda, an endogenously produced a third α chain of basement membrane collagen, type IV). It inhibited specific for the protein synthesis of endothelial cells (Maeshima et al, 2002). In the experiment on rats, it showed that Tumstatin could inhibit tumor growth (Maeshima et al, 2000), and anti-tumor activity of Tumstatin was also verified (Maeshima et al, 2002). A physician, J. Folkman in Harvard medical collage in USA firstly mentioned the theory inhibiting tumors through angiogenesis. He thought that if the blood vessels of tumors were inhibited, tumors could not get hyperplasia, metastasis instead of shrinking. Tumstatin prevents angiogenesis through inhibition of endothelial cell proliferation and promotion of apoptosis with no effect on migration, whereas endostatin prevents endothelial cell migration with no effect on proliferation. Therefore, it probably fit for curing many types of cancers. Because of the distinct properties of tumstatin and endostatin, it indicated that they had diverse antiangiogenic actions (Sudhakaret al 2003).

Up to now, few of the structure, characteristics and its’ protein knowledge of tumstatin has been known, and in particular, the report on the gene of tumstatin from Chinese human tissues has not been found yet. Additionally, Purification of bioactive recombinant protein from E. coli has been recognized challenging. Our strategy would center on the optimization of the E. coli expression system because of its higher efficiency in expressing foreign proteins as compared with the other systems. The study showed the cloning, expression, purification and its’ activity of tumstatin from Chinese kidney tissues. It would lay a theoretical foundation for the clinical application on tumstatin.

II. Materials and methods

A. Material, bacterial strains and reagents

The kidney tissue of abortus fetus were collected from associated hospital in medical college Inner Mongolia. PET-His
expressive vector, E. coli host strain, DH5α, and BL21(DE3)pLySs stored in our laboratory, RNA purified kit (Shanghai Huashun Co), pGEM-Tvector kit, T4 DNA ligase and plasmid purified kit (Promega), restriction enzyme, BamHI, Nhe I (NEB), Taq plus DNA polymerase, dNTPs, X-gal, IPTG and agarose (biotechnology Co, Shanghai), DL2000 DNA molecular weight marker and multi clone antibody (Invitrogen), HRP-labeled IgG of sheep against mouse (Huantai Co in Beijing).

B. Combined buffer
20 mmol/L NaH2PO4, 500mmol/L NaCl, pH 7.4, Washing buffer NaH2PO4, 20 mmol/L, NaCl 500 mmol/L, imidazole 500 mmol/L, pH 7.4.

C. Sequencing and cloning of Tumstatin
1. Synthesis and designing of the primers
A pair of primers was designed according to the sequence from GenBank (No. AF258351), tum1: 5’-CGGGATCCCGAGTTGAAAGG-3’ and tum2: 5’-GGCTAAGGTCTTTCATGCACA-3’, underlined nucleotides indicated the recognized sites of restriction enzyme as BamHI, NheI. Amplified the fragment of gene was about 750 nt long.

2. Preparation of template
Total RNA of kidney from Chinese abortus fetus was isolated with RNA extract kit. Reverse transcription (RT) would be carried out when the content and purity were qualified. It did according to the instruction of the RT kit. PCR would be done with cDNA synthesized as a template.

3 Cloning and sequencing of Tumstatin
SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) E. coli Top10 was grown on Luria-Bertani (LB) medium and incubated at 37°C under aeration. Amplification reactions were performed in a total volume of 50 μL containing 100 μM (each) dATP, dCTP, dGTP, and dTTP, 25 pmol of each primer, 2 ng of pLSC400 DNA, 2.5 U of Pwo DNA polymerase (Boehringer, Mannheim, Germany), and the corresponding 1x Pwo buffer. Reactions were carried out with a Perkin-Elmer thermocycler by using initial denaturation at 94°C for 5 min, followed by 5 cycles consisting of 94°C for 30s, 46°C for 30s, and 72°C for 80s and followed by 25 cycles consisting of 94°C for 30s, 55°C for 30s, and 72°C for 80s a final extension step consisting of 72°C for 10 min. The amplified products were identified by electrophoresis of 1% agarose. Each DNA was further purified by treatment with phenol-chloroform as described by Sambrook et al, 1989. Plasmid DNA was isolated from the recombinant E. coli by a method described previously (Sambrook et al, 1989). DNA sequences were determined by the dideoxy chain termination method with sequencing kits (Biotechnology Co, Shanghai). The purpose product by PCR was ligated with GEM-T vector, then transformed to E. coli DH5α competent cells by the method of CaCl2. And recombinant were selected through blue and white spots, and identified by situ-PCR and endonuclease digesting. The positive recombinant plasmid would be sequenced by Biotechnology Co, Shanghai.

D. Construction and inducing expression of pET-His-tumstatin plasmid
The extracted plasmid containing tumstatin gene, pGEM-Tum was digested with BamHI and NheI. Tumstatin DNA was recollected and cloned into expressive vector, pET-His digested with the same two enzymes, that contained an NcoI site and a PstI recognition sequence within the forward and reverse primers, respectively. The amplified product was digested with NcoI and PstI and cloned into expressive vector, pET-His digested with the same two enzymes mentioned above and ligated to generate plasmids pET-His-tumstatin. The plasmids were subsequently transferred to E. coli cells. The recombinants were selected and identified named pET-His-tumstatin. The pET-His-tumstatin were also transferred to E. coli BL21 (DE3) pLySs competent cells. and the positive bacteria were identified by PCR. The bacteria selected were incubated in LB medium induced with IPTG in different concentrations of 0.2, 0.4, 0.6, 0.8 mmol/L at the same time. And the bacteria were sampled 0.2ml once each before and after inducing. The samples were precipitated and cellular proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The quantity of expression was analyzed by Gel imaging instrument made in Japan.

E. Tumstatin purification
To determine Tumstatin activities, the engineering E. coli added in 1000 ml of LB culture with a 1/100 volume. And E. coli was grown at 37°C to an optical density of 0.5 at 570 nm. The culture was added with a final concentration of 0.2 mmol/L IPTG at 37°C for 3h. The cell culture was pelleted by centrifugation at 5000 x g for 10 min, and the cells were resuspended in 100ml of 30mM PBS buffer, and centrifugation at 3500 x g for 10 min, and resuspended as above mentioned. Cell lysis was carried out by ultrasonic way. And the cell fragments were removed by centrifugation at 13,000 x g for 30 min. The supernatant is run through a volume of 5 ml HiTrap chelating Ni-NTA column (Amersham Pharmacia). ÄKTA FPLC purifying system for protein would be connected with the column. The column is then washed with the washing buffer, followed by elution of the bound protein from the column using the elution buffer. Finally, the column is re-equilibrated with washing buffer. washing or eluting, the compounds down the column by varying the eluting solvent using a flow rate of 1ml/min. And all the fractions were pooled with an absorbance > -0.03. And the tumstatin solution was concentrated using Ultrafree-15 concentrators of 10kDa. The Ultrafree-15 concentrators are used to concentrate protein samples based on a technique known as ultrafiltration. These disposable devices hold up to 15 ml of sample at a time and can be centrifuged at 2,000 x g for 15 min, and the step was repeated for 3 times (refer to the Ultrafree-15 manual for more information). The samples were pooled and resolved with sterilized PBS of 10ml. The samples were analyzed by the gel of 15 % SDS-PAGE and also quantified with the method of Bradford.

F. Identification of Tumstatin
1. Western blot of tumstatin
After running SDS-PAGE (Sambrook et al, 1989), the extracts were transferred to nitrocellulose membrane (Sigma). Blots were stained firstly with Ponceau dye for 2 min and then developed with first antibody (antiserum against V5 from mice), followed by staining with secondary antibody (horseradish peroxidase labeled anti-mouse IgG).

2. Detection using indirect ELISA
For visualization, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used. Tumstatin (450 μg/ml) was diluted at 1:100, 1:200, 1:400 and 1:800 respectively, and added 100 μl each plate on hole of 96 holes at 4°C overnight. Next day, the plate was blocked with 3% bovine serum albumin in Tris buffered saline with 0.1% Tween 20 for 1 h and incubated with 100 μl first antibody (polyclione against-mouse antiserum) at 1:500 dilutions for 30min at 37°C. After washing with PBST, it was also incubated with 100 μl of HRP labeled anti-mouse IgG at 1:1000 dilutions and washed as above mentioned. A drop of
developing fluid A and B were added respectively for 5min followed by adding a drop of terminal reactive fluid. OD values were determined at 450nm. The positive was determined according to the ratio of experimental holes to negative holes if the ratios were larger than 2.1.

3. Antitumor effect of Tumstatin

Twenty 7-week-old male Kun Ming mice without thymus gland per group were used as test animals. And kidney tumor induction was performed as follows. 786-0 nephrosarcoma cells were subcutaneously transplanted to the back region, and attacking numbers of nephrosarcoma cell were 2×10⁶. After a week of injections, when the tumor growth volume was up to 600-700mm³, the ten mice were injected tumstatin of 6 mg/kg subcutaneously in the back region a time, and once a day for ten time injections. however, the another ten mice were only injected with 0.9% normal saline (vehicle) at the same time. Tumor growth volume (width × length ×0.52) needed to be determined with vernier caliper on a daily basis.

III. Results

A. Cloning and sequencing of tumstatin

PCR product of tumstatin would run electrophoresis using 1% agarose, the result showed the 700bp fragment presenting, and it was the same as anticipation. It found the nucleotides of the 96th was mutated T→C but nonsense mutation (Figure 1).

B. Construction of expression vector

The fragment of pGEM-T/tum-digested with BamHI and NheI was cloned into pET-His plasmid, and ligation product were transformed DH5α competent cells. Thus 5 monoclonal colony were selected and identified by PCR. DNA of positive plasmid was extracted and digested with BamHI and NheI. An objective fragment of 741 bp was finally identified (Figure 2).

C. Expression and induction of recombinant tumstatin

To investigate the regulation of tumstatin expression by IPTG and time, recombinant E.coli was each grown in three batches by shaking conditions for approximately 3h. Through several conditions optimization, concentrations of IPTG added was 0.2 mmol/L IPTG and inducing time was 3h at 30°C. The quantity of expression recombinant protein in gross protein was about 35.66. It also presented either inclusion bodies or soluble proteins (Figure 3).
**D. Purification of Tumstatin**

Purification of human tumstatin was achieved using the IMAC column with 6×His tag, and the column was washed with 200 mmol/L imidazole gradient elution buffer. 15 ml eluted fluid were obtained and concentrated. Furthermore, the absorbance of concentrated fluid at 280 nm and the method of Bradford (Kirazov et al., 1993; Liu, 2001) provided identical values for the protein concentrations (850 μg/ml). 25 mg of purified tumstatin were obtained in all, and sheet scanning of the resulting purified tumstatins indicated that the proteins were more than 95% pure (Figure 4).

**E. Identification of Tumstatin protein**

Western blot indicated visible band the position 29 kDa around (Figure 4), and also indicated the purified tumstatin protein had been recognized by specific polyclonal antibody. Furthermore, the results of indirect ELISA showed that tumstatin also could be detected when it was a 1:1000 dilution (0.085 mg). It consequently was identified the activity of tumstatin through its’ immunoreaction.

**F. In Vivo antitumor effect of Tumstatin**

To assess the antitumor activity of the obtained tumstatin, the Kun Ming mice without thymus gland were inoculated 786-O nephro sarcoma cells as carcinoma model in this study. After comparing the tumor growth volume between the experimental group and the control one, it was found that a substantial inhibitory effect was observed in mice treated with tumstatin (Fig 5), and the degree of inhibition appeared to be similar (610±98.6 mm³ in the experimental group instead 1100±155.2 mm³ in the control one). There was a significant difference between them (t test, p<0.05). These results suggest that an expression and purification system for tumstatin protein from E. coli has been successfully established in a laboratory setting.

![Figure 4. Purification and western blot of soluble Tumstatin](image)

![Figure 5. In vivo antitumor effect of tumstatin](image)
endostatin. Our results presented in the significant tumor growth 20mg/kg/d dose of purified endostatin given gives a protein has an antitumor effect in vivo at a low dos. Additionally, it showed that the human tumstatin protein from E. coli as an inclusion body form was shown. Furthermore the in vivo antitumor effect of the purified protein are comparable to those of the control, there is a significant difference. The results showed that the IPTG-inducible T7 lac promoter used in our system has previously been shown to be highly efficient in expressing heterologous proteins, including tumstatin. Additionally, with the conventional purification protocol described previously, the cultured bacteria are lysed under denaturing condition (8 M urea), and the dissolved tumstatin is then subject to bound to a Ni-NTA column chromatography for His tag-specific purification. And after the recombinant protein was extracted through the column and also passed through a step of ultrafiltration with Ultrafree-15 ultrafiltration tube (Millipore). Through these steps, a purified recombinant tumstatin could be achieved. However, the purified protein precipitates during the dialysis that eliminates urea from the solvent system. In this study, a purification approach was taken the advantages of inclusion body formation in the tumstatin-expressing E. coli cells. Usually, inclusion bodies are insoluble or biologically inactive molecules, however, in our approach, highly purified tumstatin could be dissolved and used the experiment. Although soluble endostatin prepared from a yeast system is being used in ongoing phase I clinical trials, the low yield (approximately 20 mg/liter culture) and high cost of the system have made it difficult to produce in quantities that are realistic for comprehensive clinical evaluation and application.

This study outlines a strategy for the cloning, expression and isolation of a soluble form of tumstatin. Additionally, it showed that the purified recombinant protein has an antitumor effect in vivo at a low dose level (6 mg/kg/ d). However, past report show that the 20mg/kg/d dose of purified endostatin given gives a significant tumor growth inhibition. Through comparason, the purified recombinant tumstatin is better than endostatin. Our results presented in this report offer an alternative method that will prove valuable in helping to determine the clinical activity of tumstatin. Thus, we anticipate that this recombinant tumstatin will have potency over an antitumor curing field.

The yield and purity of this antitumor protein produced from the reported procedure allow its virtual application at different laboratory levels. The established protocol also has the potential to be adapted to a larger scale production.

**References**


