ISSN 1672-9145 Acta Biochimica et Biophysica Sinica 2004, **36**(12): 845–850 **CN** 31-1940/Q

Short Communication

Recombinant Mouse Canstatin Inhibits Chicken Embryo Chorioallantoic Membrane Angiogenesis and Endothelial Cell Proliferation¤¶

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Abstract Human canstatin, a 24 kD fragment of the α2 chain of type IV collagen, has been proved to be one of the most effective inhibitors of angiogenesis and tumor growth. To investigate *in vivo* antiangiogenesis activity and *in vitro* effects on endothelial cell proliferation of recombinant mouse canstatin, the cDNA of mouse canstatin was introduced into an expression vector pQE40 to construct a prokaryotic expression vector pQE-mCan. The recombinant mouse canstatin efficiently expressed in *E. coli* M15 after IPTG induction was monitored by SDS-PAGE and by Western blotting with an anti-hexahistidine tag antibody. The expressed mouse canstatin, mainly as inclusion bodies, accounted for approximately 35% of the total bacterial proteins. The inclusion bodies were washed, lysed and purified by the nickel affinity chromatography to a purity of approximately 93%. The refolded mouse canstatin was tested on the chicken embryo chorioallantoic membranes (CAM), and a large number of newly formed blood vessels were significantly regressed. In addition, recombinant mouse canstatin potently inhibited endothelial cell proliferation with no inhibition on non-endothelial cells. Taken together, these findings demonstrate that the recombinant mouse canstatin effectively inhibited angiogenesis of the chicken embryo in a dose-dependent manner and specially suppressed *in vitro* the proliferation of human umbilical vein endothelial cells.

Key words mouse canstatin; angiogenesis inhibitor; prokaryotic expression

It is now well documented that the growth and metastasis of malignant tumors beyond a few mm^3 depend largely upon the formation of networks known as angiogenesis [1–3]. Several studies have shown that the tumor mass can be restricted to within a certain limited size by inhibiting the target of tumor-related angiogenesis. Consequently, the tumor would remain in the so-called dormant state. Obviously, tumor antiangiogenesis therapy or tumor dormancy therapy appears to be promising [4, 5].

Human canstatin [6], a fragment of α2 chain of type IV collagen, has proved to suppress endothelial proliferation *in vitro* and tumor growth *in vivo* significantly. A previous study by Kampaus [6] suggested that ED_{50} of human canstatin is approximately three times less than that of endostatin and canstatin may be more potent than endostatin in tumor dormancy therapy. In our previous study, the cDNA of mouse canstatin was cloned from total RNA of the mouse liver with 684 bp in length, encoding a 227 amino acids product [7] (GenBank accession No. AY375463). The sequences of both cDNA and amino acid share high homology with human canstatin, with identity of 89% and 96% in cDNA and amino acid sequence to human canstatin [7]. According to these observations, we postulated that mouse canstatin could be an angiogenesis inhibitor.

In order to investigate anti-angiogenesis effect of recombinant mouse canstatin, the prokaryotic expression

Received: September 22, 2004 Accepted: November 16, 2004

This work was supported by the grants from the Key Science and Technologies Program of Henan Province (No. 0122032500) and the National Natural Science Foundation of China (No. 30270031)

¤GenBank accession No. AY375463

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vector pMD18T-mCan was contructed to express recombinant mouse canstatin in *E. coli* M15 after IPTG induction. Recombinant mouse canstatin showed an inhibition effect on angiogenesis of chicken embryo CAMs and suppression of endothelial cells. This study permits further studies on its inhibitory activity of tumor growth, molecular mechanism and potential application for tumor antiangiogenesis therapy clinically.

Materials and Methods

Reagents and materials

The vector pMD18T-mCan containing the cDNA of mouse canstatin was constructed in our laboratory previously [7]. Prokaryotic expression vector pQE40, mouse anti-human hexahistidine tag antibody and Ni-NTA spin column were purchased from Qiagen. Protein refolding kit was purchased from Novagen; BCA protein assay reagent was from Pierce.

Cell lines and culture

Human umbilical vein endothelial cells (HUVEC) were purchased from American Type Collection. NIH3T3 fibroblasts, esophageal cancer cell line (Eca-109) and liver cancer cell line HepG2 were obtained from Pathology Department, Zhengzhou University. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) medium supplemented with 15% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Construction of prokaryotic expression vector pQE-mCan

The cDNA sequence encoding mouse canstatin was amplified by PCR from the vector pMD18T-mCan using follwoing primers.

Forward primer P1: 5′-CGGGATCCTTATTTGAAAAG-AAAGTGTATCTCTC-3′

Reverse primer P2: 5′-CCCAAGCTTATCAAATGA-AGGGGCCGCACACTGAGG-3′

The *Bam*HI site in forward primer P1, and the *Hin*dIII site in reverse primer P2 were underlined respectively. PCR conditions consisted of 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1.5 min. The PCR product was separated on 1% agarose gel containing ethidium bromide (EB) and visualized by UV-light illumination. The amplified cDNA fragment was inserted into prokaryotic pQE40 to construct an *E. coli* expression vector pQE-mCan.

Expression, purification of mouse canstatin in *E. coli* M15

The constructed expression vector pQE-mCan was transformed into *E. coli* M15 for expression. *E. coli* M15 was cultured in LB medium for approximately 2 h until the culture reached an A_{600} of 0.6. Subsequently, recombinant mouse canstatin expression was induced by the addition of IPTG to a final concentration of 0.8 mM. After a 4 h induction, *E. coli* M15 cells were harvested by centrifugation at 5000 *g*, lysed by using the lysing buffer containing 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0, and sonicated briefly. The mixture was centrifuged at 8000 *g* for 30 min. The supernatant fraction was applied onto the Ni-NTA spin column, and nonspecifically bound proteins were removed by washing with 20 mM imidazole in 8 M urea, 0.1 M NaH_2PO_4, 0.01M Tris-HCl, pH 8.0. The recombinant protein with His_6-tag was eluted by 250 mM imidazole in 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0, and refolded by dialysis against 20 mM Tris-HCl, pH 8.5 and 1 mM reduced glutathione, 0.2 mM oxidized glutathione in 20 mM Tris-HCl, pH 8.5. Protein concentration was determined by the bicinchoninic acid (BCA) assay. Refolded recombinant mouse canstatin was ready for use of the following experiments. All the above procedures were performed as described previously [8].

Western blotting

Western blotting was performed as follows. Total protein of *E. coli* M15 was run on 12% SDS-polyacrylamide gel and then transferred onto nitro-cellulose membrane. Blots were hybridized with mouse anti-hexahistidine monoclonal antibody for overnight. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody and visualized with the Amersham enhanced chemiluminescence ECL system.

Chicken embryo chorioallantoic membrane (CAM) assay

The CAM assay was conducted according to the method described previously [9] with some modifications. The air chamber of 4-day-old chicken embryo was carefully removed, and the recombinant mouse canstatin dried on filter paper of 1 mm×1 mm was implanted onto the CAM of individual embryo. After incubation for 48–72 h at 37 °C, CAMs were examined for the formation of avascular zones.

Endothelial cell proliferation assay

Human umbilical vein endothelial cells (HUVECs),

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NIH3T3 fibroblasts, esophageal cancer cell line Eca-109 cells, and liver cancer cell line HepG2 cells were grown to 90% confluence in DMEM with 15% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were harvested by trypsinization at 37 °C for 5 min. A suspension of approximately 3000–5000 cells in 100 μl DMEM supplemented with 5% FCS was added in triplicate into each well of 96-well tissue culture plate, and incubated for 24 h at 37 °C with 5% CO_2 and 95% humidity. Then the culture medium was removed and replaced with 150 μl fresh DMEM containing 5% FCS. Other cells, used as controls, were also grown to confluence, trypsinized and plated in the same manner. Mouse canstatin dissolved in 50 μl PBS at different concentration was added into each well, whilea the blank control wells were added with equal volume of BPS. After 72 h incubation, 20 μl 3-(4,5-dimethylthiazolzyl) 2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for another 4 h at 37 °C with 5% CO_2, and 95% humidity. After the reaction, the medium was pipetted out from each well and 150 μl DMSO was added. The absorbance A_{570}, which correlates to the number of cells, was measured on a microplate reader at 570 nm wavelength.

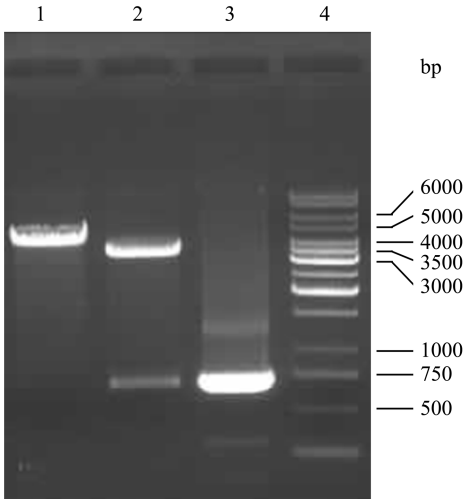

Fig. 1 Restriction enzyme analysis of prokaryotic expression vector pQE-mCan

1, pQE-mCan was digested by *Bam*HI; 2, pQE-mCan was digested by *Bam*HI and *Hin*dIII; 3, PCR product on a template of pQE-mCan; 4, DNA marker (Gene Ruler 1000 bp ladder).

Results

Construction of prokaryotic expression vector pQE-mCan

The vector pMD18T-mCan constructed previously was used to amplify the cDNA of mouse canstatin with *Bam*HI and *Hin*dIII restriction enzyme site at the 5′ and 3′ end respectively by PCR. Analysis of 1% agarose gel showed that the resulting fragment was approximately 700 bp in length.

The amplified mouse canstatin cDNA was subcloned into the prokaryotic expression vector pQE40, which was identified by the double digestion of restriction enzyme *Bam*HI and *Hin*dIII and sequencing. The results revealed that the cDNA for mouse canstatin was correctly inserted between *Bam*HI and *Hin*dIII site of pQE40 (Fig. 1).

Expression and purification of recombinant mouse canstatin in *E. coli* M15

After the induction of 0.8 mM IPTG at 37 °C, SDS-PAGE analysis revealed a monomeric band at approximate 28 kD which was estimated to be the recombinant mouse canstatin and accounted for approximately 35% of the total protein of *E coli* M15 (Fig. 2). Most of the recombinant protein was found in the insoluble fraction (inclusion bodies). The inclusion bodies were washed, lysed and centifuged. The supernant was applied to the Ni-NTA spin column, and the recombinant protein was purified to a purity of 93% by the affinity chromatography under denaturing condition. The purified recombinant mouse canstatin was then refolded by the dialysis against a variety of dialysis buffers. Some of the protein was precipitated during the refolding process, and the protein present in solution was used for *in vivo* CAM assay.

Identification of recombinant mouse canstatin generated from *E. coli* M15

To characterize recombinant mouse canstatin, Western blotting was carried out using mouse anti-hexahistidine monoclonal antibody to demonstrate the presence of recombinant mouse canstatin with a His tag (Fig. 3). Recombinant mouse canstatin on membrane reacted with the specific antibody. The proteins had molecular weigh of approximately 28 kD which is a little larger than the size of mouse canstatin (24 kD) due to bacterial-expressed mouse canstatin as a fusion protein with an N-terminal six histidine tag.

Chicken embryo chorioallantoic membrane (CAM) assay

To investigate the *in vivo* antiangiogenic activity of the

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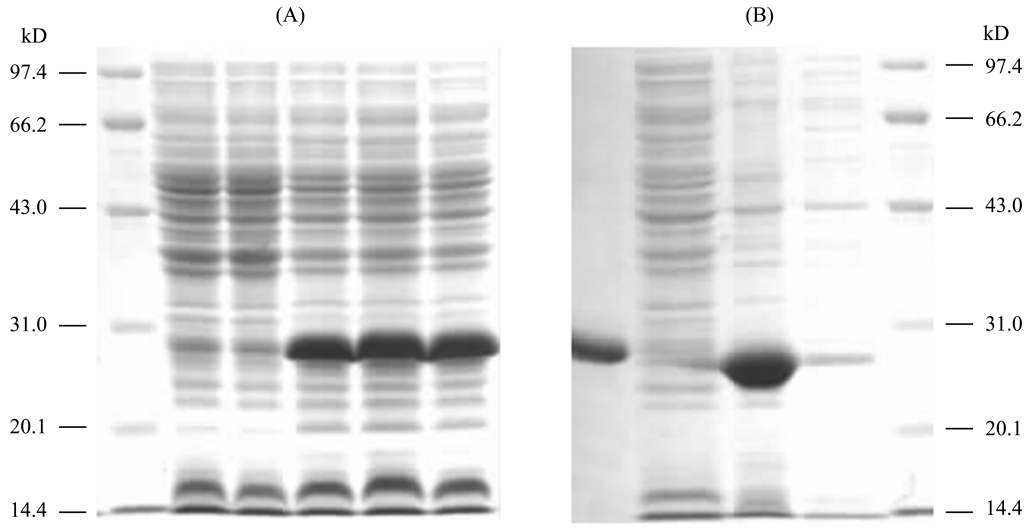

Fig. 2 Expression of mouse canstatin in *E. coli* M15 and its purification

(A) Expression of recombinant mouse canstatin. 1, protein molecular weigh marker; 2 and 3, before IPTG induction; 4, 5 and 6, 3, 4 and 5 h after IPTG induction respectively. (B) Purification of recombinant mouse canstatin. 1, mouse canstatin after purification by Ni-NTA spin column; 2, before IPTG induction; 3, inclusion body; 4, supernatant after sonication of bacteria; 5, protein molecular weight marker.

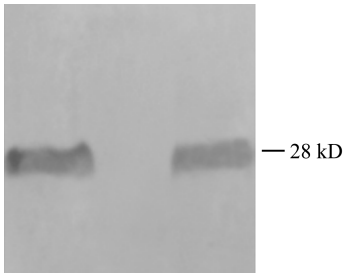

Fig. 3 Western blot analysis of the recombinant mouse canstatin from *E. coli* M15

The molecular weigh of recombinant mouse canstatin was approximately 28 kD.

recombinant mouse canstatin, the CAM assay was performed. 5–25 μg mouse canstatin per embryo was found to inhibit the new embryonic blood vessel growth by measuring avasular zone [Fig. 4(B)]. Within the avascular zone, a large number of newly formed blood vessels were significantly regressed. The inhibition effect was found to be dose-dependent over the range of 5–25 μg canstatin per embryo, and no obvious inflammation was detected. However no significant antiangiogenesis was observed in the control embryos implanted with filter paper soaked in PBS alone [Fig. 4(A)]. These results indi-

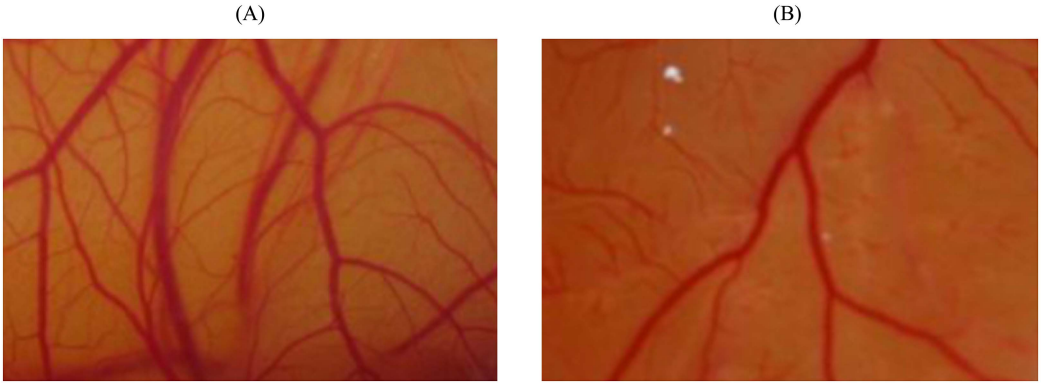

Fig. 4 Inhibition of angiogenesis by recombinant mouse canstatin on CAM

(A) A control CAM assay with filter paper soaked in PBS alone. (B) A CAM assay with filter paper containing recombinant mouse canstatin.

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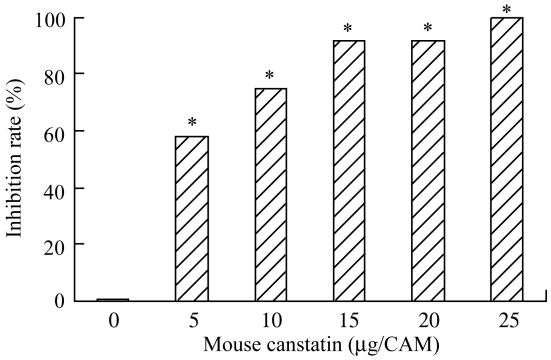

Fig. 5 The inhibition rate at different concentration of mouse canstatin

The data are shown as the percentage of CAM number with avascular zones (n=12), n represents the sample number of each tested group. *P<0.005 vs. the control group treated with PBS alone.

cate that mouse canstatin is able to suppress angiogenesis on chicken embryo CAM (Fig. 5).

Inhibition effect of recombinant mouse canstatin on endothelial cell proliferation

We examined the inhibitory effect of recombinant mouse canstatin on the proliferation of human umbilical vein endothelial cells. As shown in Fig. 6 and Fig. 7, the recombinant mouse canstatin significantly inhibited the proliferation of endothelial cells, but no significant effect was observed on the proliferation of all examined non-endothelial NIH3T3, Eca-109 and HepG2 cells. These results demonstrated that the recombinant mouse canstatin specifically

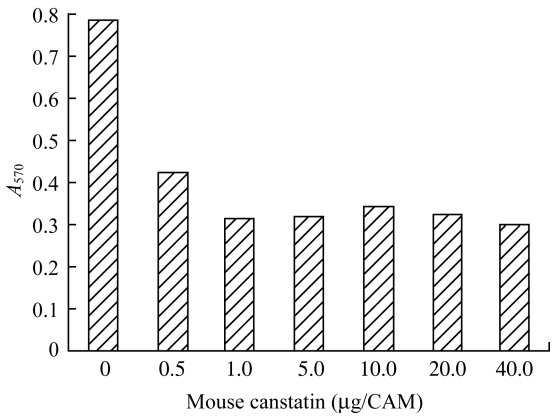

Fig. 6 Effect of mouse canstatin on the proliferation of endothelial cells

Values represent means of four determinations (n=4) by MTT assay.

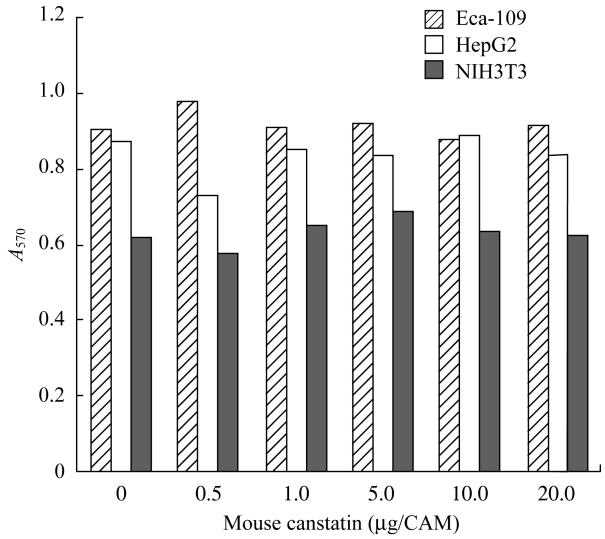

Fig. 7 Effect of mouse canstatin on the proliferation of non-endothelial cells

Values represent means of four determinations (n=4) by MTT assay.

inhibited the proliferation of endothelial cells.

Discussion

Angiogenesis is required for tumor growth and the possibility of inhibiting tumor growth by blocking the formation of new tumor vessels has recently received attention because of the direct endothelial targeting and the absence of drug resistance. It has been demonstrated that a variety of endogenous angiogenesis inhibitors such as angiostatin [10], endostatin [11], restin [12,13], and tumstatin [14, 15] could lead to the suppression of primary and metastatic tumor growth without affecting the normal vasculature growth. Human canstatin is one of recently discovered angiogenesis inhibitor which can inhibit endothelial cells proliferation with no effect on tumor cell growth *in vitro* and suppress the growth of implanted PC-3 human prostate carcinoma and 768-0 renal cell carcinoma cells in severe combined immunodeficiency and athymic nude mice, respectively. The mechanism by which human canstatin inhibits Akt activation and induces Fas-dependent apoptosis in cultured human umbilical vein endothelial cells has been demonstrated [16].

In the present study, recombinant mouse canstatin was expressed in *E. coli* M15 and analyzed by SDS-PAGE. Western blotting with an anti-hexahistidine tag also detected bacterially expressed recombinant mouse canstatin, because it was produced in *E. coli* M15 as a fusion pro-

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tein with an N-terminal histidine tag. Mouse canstatin was isolated from the host cells by affinity chromatography techniques. Refolding reagents were used to recover the activity of recombinant mouse canstatin. As showed above, mouse canstatin produced in *E. coli* M15 inhibited newly formed blood vessels in a dose-dependent manner, with a near-complete inhibition of CAMs seen with the addition of 20 μg/CAM of mouse canstatin. In addition, recombinant mouse canstatin significantly inhibited endothelial cells with no significantly effects was observed on the proliferation of all examined no-endothelial cells such as NIH3T3, Eca-109 and HepG2. These results demonstrated that recombinant mouse canstatin specially inhibited the proliferation of endothelial cells. Collectively, these observations support the conclusion that well-folded mouse canstatin expressed in bacteria may be an effective anti-angiogenic molecule with potent activities as has been demonstrated for mouse canstatin. These discoveries may add to our understanding of antiangiogenesis effects of mouse canstatin.

Further studies with recombinant mouse canstatin are underway to test its inhibition effect on tumor growth *in vivo*. Future researches may give insight into its unique molecular mechanisms underlying the angiostatic actions of recombinant mouse canstatin. Therefore, the recombinant mouse canstatin may be a promising canditate in cancer therapy.

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