Angiostatic Effects on Chick Chorioallantoic Membrane by Recombinant N-Fragment of Mouse Canstatin*

HOU Wei-Hong, JIA Yan-Long, WANG Tian-Yun, CHAI Yu-Rong,
YUAN Bao-Mei, TIAN Fang, WANG Jian-Min, XUE Le-Xun**
(*Laboratory for Cell Biology, Zhengzhou University, Zhengzhou* 450052, China)

Abstract Mouse canstatin is a C-terminal globular non-collagenous domain of type Ⅳ collagen α2 chain, which previously showed anti-angiogenic activity. To investigate *in vivo* angiostatic effects on chick chorioallantoic membrane (CAM) by recombinant mouse canstatin N-fragment(1 ~ 95aa), the cDNA for N-fragment of mouse canstatin, obtained from a cloning vector pMD18T-mCan by PCR, was introduced into an expression vector pET30a(+) to construct prokaryotic expression vector pET-mCanN. N-fragment of mouse canstatin efficiently expressed in *E. coli* BL21(DE3) after IPTG induction was monitored by SDS-PAGE and by Western blotting with an anti-hexahistidine tag antibody. The expressed N-fragment of mouse canstatin, mainly as inclusion bodies, accounted for approximately 18% of the total bacterial proteins as estimated by densitometry. The inclusion bodies were washed, lysed and purified by the nickel affinity chromatography to a purity of 92%. The refolded N-fragment of mouse canstatin was tested on the chicken embryo CAMs, and a large number of newly formed blood vessels were significantly regressed. It is concluded that N-fragment derived from mouse canstatin is an effective inhibitor of angiogenesis of the chicken embryo in a dose-dependent manner through CAM assay and a promising candidate for the treatment of cancer, suggesting that these novel findings may add to the understanding of anti-angiogenesis effects of the N-fragment of mouse canstatin.

Key words canstatin, tumor angiogenesis inhibitor, prokaryotic expression

Malignant tumor growth is an angiogenesis-dependent process[1~3] and the restriction of angiogenesis may be a new valuable approach to cancer therapy. By inhibiting the target of tumor-related angiogenesis, the tumor mass can be restricted to within a certain limited size. Consequently, the tumor would remain in the so-called dormant state. Obviously, tumor anti-angiogenesis therapy or tumor dormancy therapy appears to be promising[4,5].

Human canstatin[6], a novel inhibitor of angiogenesis derived from the C-terminal globular non-collagenous domain (NC1) of α2 chain of type Ⅳ collagen, has been proved to suppress endothelial proliferation *in vitro* and tumor growth *in vivo* significantly. The previous studies by Kampaus 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[6]. In our previous study, the cDNA of mouse canstatin was cloned from total RNA of the mouse liver with 684 bp in length, encoding 227 amino acids. The sequences of both cDNA and amino acid share high homology with human canstatin, with identity 89% cDNA and 96% amino acids to human canstatin. In addition, N-fragment(1 ~ 95aa) of mouse canstatin, sharing 100% homology of amino acid sequences with that of human canstatin, was predicted to be a potent angiogenisis inhibitor. In order to investigate anti-angiogenesis of recombinant N-fragment of mouse canstatin, the prokaryotic express vector pET30a(+) was used to express N-fragment of mouse canstatin in *E. coli* BL21(DE3) after IPTG induction. Recombinant N-fragment of mouse canstatin after isolation, purification and refold showed inhibition of angiogenesis on chicken embryo CAMs. This study permits further study on its inhibitory activity of tumor growth, molecular mechanism and potentially clinical application for tumor anti-angiogenesis therapy.

1 Materials and methods

1.1 Materials

The vector pMD18T-mCan containing the cDNA for mouse canstatin was constructed in our laboratory previously. Prokaryotic expression vector pET30a(+) was purchased from Invitrogen. Mouse anti-human hexahistidine tag antibody and Ni-NTA spin columns were obtained from QIAGEN. Protein refolding kit were purchased from Novagen. BCA protein assay reagents were from PIERCE.

1.2 Methods

1.2.1 Construction of prokaryotic expression vector

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** Corresponding author.
Tel: 86-371-6658332, Fax: 86-371-6997182
E-mail: lxxue@public2.zz.ha.cn

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pET-mCanN: Prokaryotic expression vector pET-mCanN was constructed by subcloning the fragment containing the cDNA for N-fragment of mouse canstatin. The cDNA sequence encoding N-fragment of mouse canstatin was amplified by PCR from the vector pMD18T-mCan using a forward primer (P1: 5′ CGGGATCCTTATTTGAAAAGAAAGTGTATCTCTC 3′) and reverse primer (P2: 5′ CCCGAGCTCCTCAGCCACGGGCATCAT 3′), in which *Bam*H Ⅰ and *Sac* Ⅰ sites exist at the ends of N-fragment of mouse canstatin cDNA. PCR conditions consisted of 30 cycles of 94℃ for 0.5 min, annealing at 55℃ for 0.5 min, and extension at 72℃ for 1.5 min. The PCR-amplified DNA fragment was separated on 1% agarose gel containing ethidium bromide (EB) and visualized by UV-light illumination. The amplified cDNA fragment was inserted into an *E. coli* expression vector pET30a(+) to construct an expression vector pET-mCanN.

1.2.2 Expression, purification and refolding of N-fragment of mouse canstatin in *E. coli* BL21(DE3): The constructed expression vector pET-mCanN was transformed into *E. coli* BL21(DE3) for expression. *E. coli* BL21(DE3) was cultured in LB medium for approximately 2 h until A_{600} of the cell culture reached 0.6. Subsequently, N-fragment of mouse canstatin expression was induced by addition of IPTG to a final concentration of 0.8 mmol/L. After induction for 4 h, recombinant N-fragment of mouse canstatin was separated on SDS-PAGE for detecting its expression. Induced *E. coli* BL21(DE3) cells were harvested by centrifugation at 5 000 *g*. The expression proteins were isolated predominantly from inclusion bodies which would be washed and lysed, and resuspended cells were sonicated briefly and centrifuged at 8 000 *g* for 30 min. The supernatant fraction was passed over Ni-NTA spin column and nonspecifically bound proteins were removed by washing. The soluble proteins were purified by Ni-NTA spin column, and then refolded by dialysis in different dialysis buffers. Protein concentration in each fraction was determined by the BCA assay. Refolded N-fragment of mouse canstatin were ready for use in the following experiments. All the procedures were performed as described previously[7].

1.2.3 Western blotting. Western blotting was performed as follows: the mouse anti-hexahistidine monoclonal antibody contributed to identify expression of the recombinant N-fragment of mouse canstatin in *E. coli* BL21(DE3). Total proteins of *E. coli* BL21(DE3) were run on 12% SDS-polyacrylamide gels and then transferred onto nitro-cellulose membranes. Blots were hybridized overnight and probed with mouse anti-hexahistidine monoclonal antibody. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized with the Amersham enhanced chemiluminescence ECL system.

1.2.4 Chicken embryo chorioallantoic membrane (CAM) assay: The CAM assay was conducted using the improved CAM technique as described[8]. The air chambers of four-day-old chicken embryos were carefully removed, recombinant N-fragment of mouse canstatin dried on filter paper of 1 mm × 1 mm were implanted on the CAMs of individual embryos. After incubation for 48 ~ 72 h, CAMs were examined for the formation of avascular zones.

2 Results

2.1 PCR amplification

The vector pMD18T-mCan constructed in our laboratory previously was used to amplify the cDNA for N-fragment of mouse canstatin with *Bam*H Ⅰ and *Sac* Ⅰ restriction enzyme sites at the 5′ and 3′ end respectively by PCR. Analysis of 1% agarose gel showed that the resulting fragment was approximately 290 bp in length (Figure 1). GenBank accession number of the cDNA for N-fragment of mouse canstatin is AY502946.

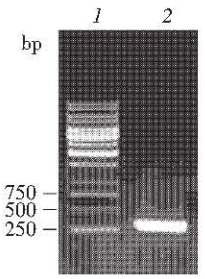

Fig. 1 PCR amplification of the cDNA for N-fragment of mouse canstatin from pMD18T-mCan

1: DNA marker(Gene Ruler 1 000 bp ladder); *2*: PCR product.

2.2 Construcuion of prokaryotic expression vector pET-mCanN

The amplified cDNA for N-fragment of mouse canstatin was subcloned into a prokaryotic expression

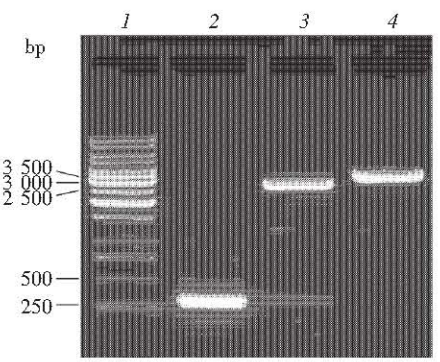

Fig. 2 Restriction analysis of a prokaryotic expression vector pET-mCanN

1: DNA marker (Gene Ruler 1 000 bp ladder); *2*: PCR product of pET-mCanN; *3*: pET-mCanN was digested by *Bam*H Ⅰ and *Sac* Ⅰ; *4*: pET-mCanN was digested by *Bam*H Ⅰ.

vector pET30a(+), subsequently identified by digestion with restriction enzymes *Bam*H Ⅰ and *Sac* Ⅰ and by PCR amplification. The results revealed that The cDNA for N-fragment of mouse canstatin was correctly inserted between *Bam*H Ⅰ and *Sac* Ⅰ sites of a prokaryotic expression vector pET30a(+) (Figure 2).

2.3 Expression, purification and refolding of pET-mCanN in *E. coli* BL21(DE3)

Recombinant N-fragment of mouse canstatin was expressed at a high level after 0.8 mmol/L IPTG induction at 37℃ in *E. coli* BL21(DE3), SDS-PAGE analysis revealed a monomeric bond at approximately 18 ku and the expressed N-fragment of mouse canstatin accounted for approximately 18% of the total bacterial proteins (Figure 3a). Most of the proteins were in insoluble fraction (inclusion bodies) from bacteria (Figure 3b). Inclusion bodies were purified using Ni-NTA spin column under denaturing conditions, and the inclusion bodies were washed, lysed and purified by the nickel affinity chromatography to a purity of 92% (Figure 3b). The purified recombinant N-fragment of mouse canstatin was refolded by dialysis in a variety of dialysis buffers according to the procedures described above. However, some of the protein precipitated out of solution during the refolding process and the proteins present in solution were used for *in vivo* CAM assay.

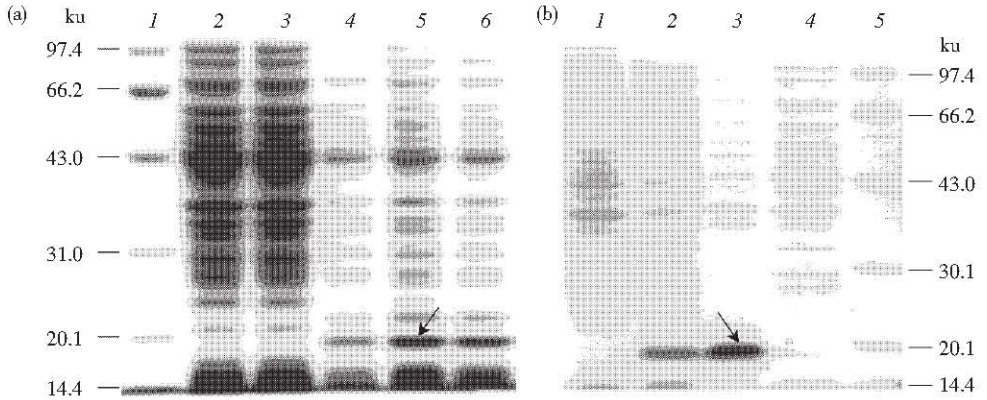

Fig. 3 Expression of N-fragment of mouse canstatin in *E. coli* BL21(DE3) and its purification

(a) *1*: Protein molecular mass marker; *2* and *3*: Before IPTG induction; *4*, *5* and *6*: 3, 4 and 5 h after IPTG induction respectively. (b) *1*: Before IPTG induction; *2*: Inclusion body; *3*: N-fragment of mouse canstatin after purification by Ni-NTA spin column; *4*: Supernatant after sonication of bacteria; *5*: Protein molecular mass marker.

2.4 Characterization of recombinant N-fragment of mouse canstatin generated from *E. coli* BL21 (DE3)

To characterize recombinant N-fragment of mouse canstatin, Western blotting was carried out using mouse anti-hexahistidine monoclonal antibody to demonstrate the presence of recombinant N-fragment of mouse canstatin with a His. tag (Figure 4). Recombinant N-fragment of mouse canstatin on membrane reacted with the specific antibody. The proteins had molecular mass of approximately 18 ku, which is a little larger than the size of N-fragment of mouse canstatin (12 ku) due to bacterial-expressed N-fragment of mouse canstatin as a fusion protein with an N-terminal six histidine tag.

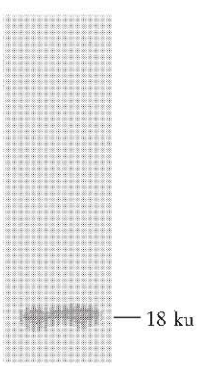

Fig. 4 Western blotting of recombinant N-fragment of mouse canstatin from *E. coli* BL21(DE3)

The molecular mass of recombinant N-fragment of mouse canstatin was approximately 18 ku.

2.5 Chicken embryo chorioallantoic membrane (CAM) assay

To investigate the *in vivo* anti-angiogenic activity, recombinant N-fragment of mouse canstatin was tested on the chicken embryo CAMs. At concentrations of 10 ~ 25 μg per embryo N-fragment of mouse canstatin inhibited new embryonic blood vessels growth, as measured by the formation of avasular zones (Figure 5b). Within the avascular zones, a large number of newly formed blood vessels were significantly regressed. The measured inhibition was dose-dependent over the range of 10 ~ 25 μg per embryo (Figure 6), with a near-complete inhibition of CAMs seen with 20 μg per embryo N-fragment of mouse

canstatin, and no obvious inflammation was detected. However no significant anti-angiogenesis was observed in the control embryos implanted with filter paper soaked in PBS alone (Figure 5a). These results indicate that N-fragment of mouse canstatin is able to suppress angiogenesis on chicken embryo CAMs ($P < 0.005$ compared with PBS alone).

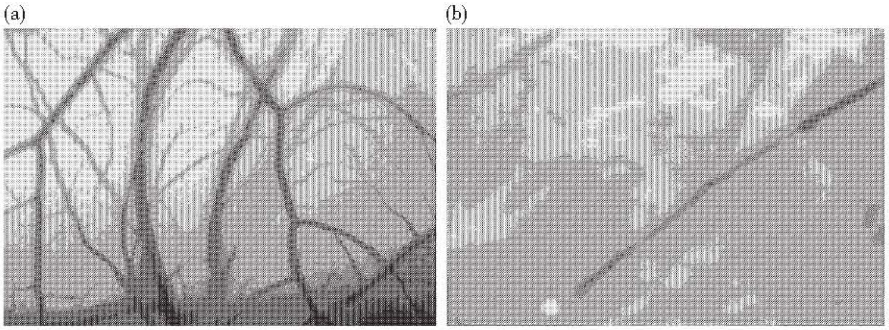

Fig. 5 Inhibition of angiogenesis by recombinant N-fragment of mouse canstatin on the CAMs

(a) A control CAM assay with filter paper soaked in PBS alone; (b) A CAM assay with filter paper containing recombinant N-fragment of mouse canstatin.

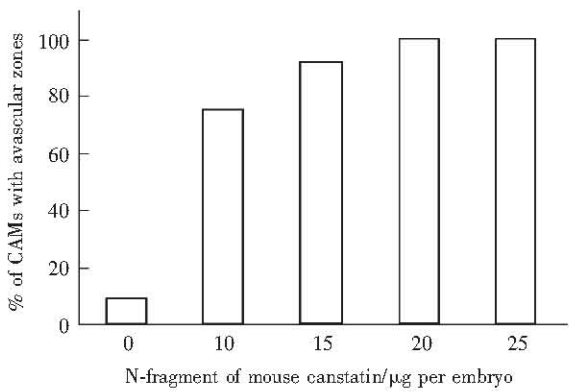

Fig. 6 The inhibition rate at different concentrations of N-fragment of mouse canstatin

The data are shown as the percentage of CAM number with avascular zones. $n = 12$, n represents the CAM number of each tested group. $P < 0.005$ compaired with PBS alone.

3 Discussion

Angiogenesis, the development of new blood vessels from pre-existing ones, is required for tumor growth and metastasis[4]. The possibility of inhibiting tumor growth by blocking the formation of new tumor vessels has recently received much attention. Anti-angiogenic tumor therapies have recently attracted intense interest because of their direct endothelial targeting and the absence of drug resistance. It has been demonstrated that a variety of endogenous angiogenesis inhibitors such as angiostatin[9], endostatin[10], restin[11,12], and tumstatin[13~15] 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 *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. Moreover, collective data suggest that canstatin could be more potent than endostatin. Our previous findings demonstrated that the recombinant mouse canstatin produced in *E. coli* effectively inhibited angiogenesis of the chicken embryo in a dose-dependent manner through CAM assay and may be a promising condicate for cancer dormany therapy.

Previous studies on tumstatin, the C- terminal globular non-collagenous domain(NC1) of α3 chain of type Ⅳ collagen, showed the anti-angiogenic activity of tumstatin was localized to the putative 54 ~ 132 amino acids (Tum-5). The recombinant Tum-5 produced in *E. coli* inhibited proliferation and caused apoptosis of endothelial cells with no significant effect on nonendothelial cells. Tum-5, at least 10-fold more active than human endostatin, is more potent than full length tumstatin. We previously showed that N-fragment(1 ~ 95aa) of mouse canstatin, sharing 100% homology of amino acid sequences with that of human canstatin, was thought to be more potent than full length mouse canstatin. In the present study, recombinant N-fragment of mouse canstatin was expressed in *E. coli* BL21(DE3) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Western blotting with an anti-hexahistidine tag also detected bacterially expressed recombinant N-fragment of mouse canstatin, because it was produced in *E. coli* BL21 (DE3) as a fusion protein with a N-terminal histidine tag using a prokaryotic expression vector pET-mCanN. In addition, N-fragment of mouse canstatin was isolated from the host cells by affinity chromatopraphy

techniques. Refolding reagents were used to recover the activity of recombinant N-fragment of mouse canstatin. As showed above, N-fragment of mouse canstatin produced in *E. coli* BL21(DE3) inhibited in a dose-dependent manner, with a near-complete inhibition of CAMs seen with the addition of 20 μg of N-fragment of mouse canstatin. Taken together, these observations support the conclusion that well-folded N-fragment of mouse canstatin expressed in bacteria may be an effective anti-angiogenic molecule with potent activities as has been demonstrated for N-fragment of mouse canstatin. These novel discovery may add to our understanding of anti-angiogenesis effects of N-fragment of mouse canstatin.

Further studies with bacterial-expressed N-fragment of mouse canstatin are underway to test the ability to inhibit endothelial cell proliferation, migration and anti-tumor growth *in vivo*. Future researches may give insight into its unique molecular mechanisms underlying the angiostatic actions of N-fragment of mouse canstatin. Therefore, N-fragment of mouse canstatin may become useful for potential clinical treatment of cancer patients.

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重组小鼠 canstatin N 端片段对鸡胚新生血管生成的抑制作用*

侯卫红 贾岩龙 王天云 柴玉荣 袁保梅 田 芳 王建民 薛乐勋**

（郑州大学医学院细胞生物学研究室，郑州 450052）

摘要 为了研究重组小鼠 canstatin N 端片段的体内抗血管生成活性，通过 PCR 扩增小鼠 canstatin N 端片段 cDNA，定向克隆于原核表达载体 pET30a（+）中，构建小鼠 canstatin N 端片段重组表达载体 pET-mCanN，转化 *E. coli* BL21（DE3），IPTG 诱导表达，SDS-聚丙烯酰胺凝胶电泳（SDS-PAGE）和蛋白质印迹检测小鼠 canstatin N 端片段的表达. 结果表明，IPTG 诱导原核表达载体 pET-mCanN 在大肠杆菌 *E. coli* BL21（DE3）中高效表达，小鼠 canstatin N 端片段表达量约占菌体总蛋白量的 18%，小鼠 canstatin N 端片段主要以包涵体形式存在，包涵体经过洗涤、裂解、Ni-spin column 亲合柱层析以及蛋白质复性等步骤纯化后，获得了纯度约为 92% 的重组小鼠 canstatin N 端片段. 鸡胚绒毛尿囊膜（chicken embryo choriollantoic membrane，CAM）实验表明，原核表达的小鼠 canstatin N 端片段能有效地按剂量依赖的方式抑制鸡胚新生血管的形成.

关键词 canstatin，肿瘤血管生成抑制剂，原核表达

学科分类号 R730.23

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** 通讯联系人. Tel：0371-6658332，Fax：0371-6997182，E-mail：lxxue@public2.zz.ha.cn

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