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# High-level synthesis of recombinant murine endostatin in Chinese hamster ovary cells

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**Abstract**

Endostatin, a carboxy-terminal fragment of collagen XVIII, has been shown to act as an anti-angiogenic agent that specifically inhibits proliferation of endothelial cells and growth of various primary tumors. Here, we describe the expression by Chinese hamster ovary (CHO) cells of murine endostatin and of a tagged-fusion protein, $(his)_6$-met-endostatin. A dicistronic mRNA expression vector was utilized in which endostatin cDNA was inserted upstream of the amplifiable marker gene, dihydrofolate reductase (DHFR). After transfection of the expression vectors, stepwise increments in methotrexate levels in the culture medium were applied, promoting gene amplification and increasing expression levels of the proteins of interest. The expression level of secreted native endostatin was about 78 μg/mL while the one for secreted $(his)_6$-met-endostatin was about 114 μg/mL, for the best expressing clones. Characterization of physico-chemical and immunological activities of the proteins was performed using SDS–PAGE and Western blotting. The biological activities of recombinant endostatins were tested with a cow pulmonary artery endothelial (C-PAE) cell proliferation assay. Both recombinant endostatin and $(his)_6$-met-endostatin inhibited, in a dose-dependent fashion, growth of C-PAE cells stimulated by basic fibroblast growth factor (bFGF).


*Keywords:* Endostatin; Angiogenesis; CHO cell; Dicistronic vector; DHFR

Angiogenesis, the formation of new blood vessels from an existing microvascular system, plays a critical role not only during embryonic development, but also during subsequent tissue growth and regeneration, and growth of tumors [1,2]. When a tumor size exceeds 2–3 $mm^3$, recruitment of new blood vessels is required to prevent tumor cell death resulting from hypoxia and nutrient deficiency [3]. Tumor cells promote angiogenesis by secretion of angiogenic factors, in particular, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which are mitogenic for endothelial cells [4,5].

Endostatin is a 20 kDa COOH-terminal fragment of collagen XVIII, produced by a murine hemangioendothelioma, which specifically inhibits endothelial cell proliferation and potently interferes with angiogenesis and tumor growth [6]. Inhibition of tumor growth of murine tumors and their metastases in animal models following administration of high levels of recombinant endostatin (10–20 mg/Kg/day) has been reported [6–8]. Expression of a fusion protein, containing the amino acid sequence MARRASVGTDHHHHHHM followed by murine endostatin, as inclusion bodies in *Escherichia coli* has first been described by O'Reilly [6]. Purification and refolding methods were developed to obtain a soluble endostatin from inclusion bodies expressed by *E. coli* cells with yields of 16 mg/L [9] and 150 mg/L [10]. Recombinant murine endostatin was also expressed in *E. coli* cells as the authentic protein secreted into the culture medium, with a yield of up to 40 mg/L. This was achieved by fusion of an alkaline phosphatase signal

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peptide with endostatin coding sequences [11]. The systems described above usually lead to insoluble or highly contaminated preparations, difficult to purify. A yeast expression system has also been used for expression of soluble endostatin with a yield of about 15–20 mg/L [12]. Finally, the production of recombinant endostatin from stably transformed *Drosophila melanogaster* S2 cells with a yield of 24 mg/L cultures has been reported [13].

Expression of heterologous genes by mammalian cells are systems in which soluble recombinant proteins with the right conformation can commonly be obtained. In addition, serum-free culture medium can be used to obtain expression of high levels of recombinant proteins with fewer contaminants. However, stable transfections of mammalian cells for the generation of high levels of murine endostatin have not yet been reported.

In the present study, expression vectors were constructed for the synthesis and secretion of both endostatin and $(his)_6$-met-endostatin by dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary (CHO) cells. The vectors, pED-endo and pED-$(his)_6$-met-endo, are dicistronic expression vectors in which the endostatin gene was inserted upstream of the EMCV leader sequence, followed by the selectable and amplifiable marker gene, DHFR. Selection for resistance to increasing concentrations of methotrexate, a DHFR inhibitor, resulted in increasing expression levels of endostatin and tagged-endostatin via amplification of DHFR and endostatin genes in the chromosome. The proper folding and secretion of the two proteins were assured by their fusion to a murine immunoglobulin signal peptide. The biological activities of the recombinant proteins were demonstrated in vitro using a C-PAE (cow pulmonary artery endothelial) cell proliferation assay.

## Materials and methods

### *Construction of expression vectors*

The pETKH-1 vector containing the murine endostatin cDNA was obtained from ATCC (ATCC No. 63404). The endostatin cDNA was first cloned in the vector pSecTag2 from Invitrogen (Carlsbad, CA, USA) containing the sequences for murine Ig κ-chain V-J2-C signal peptide. Purified pETKH1 was digested with the restriction enzyme, *Nhe*I, followed by treatment with Klenow fragment of DNA polymerase I to obtain blunt ends, and by digestion with the enzyme *Kpn*I. The DNA fragment (0.6 kb) containing the endostatin cDNA was cloned into the pSecTag2 vector digested with restriction enzymes *Kpn*I and *Eco*RV. Removal of nucleotides located between the leader sequence and the codon for the first amino acid of endostatin was performed using site-specific mutagenesis by the method of Kunkel [14]. The 5′-phosphorylated oligonucleotide used in the mutagenesis was: 5′TGGCTGAAAGTCCTGATGAGTAT GACCAGTGGAACCTGGAACCCA 3′. Another vector containing the leader sequence followed in-frame by sequences coding for $(his)_6$-met-endostatin was obtained by site-specific mutagenesis with the 5′-phosphorylated oligonucleotide: 5′TGGCTGAAAGTCCTGATGAGT ATGCATATGGTGGTGGTGATGGTGGTCACCA GTGGAACCTGGAAC 3′. Positive clones were sequenced. DNA-containing sequences for signal peptide plus endostatin cDNA or signal peptide plus $(his)_6$-met-endostatin were then cloned in the vector, pED, kindly provided by Dr. R.J. Kauffman (Howard Hughes Medical Institute, University of Michigan). Vectors pSecTag2-endo and pSecTag2$(his)_6$-met-endo were digested with the restriction enzyme *Xho*I, followed by treatment with Klenow fragment of DNA polymerase I to obtain blunt ends and by the digestion with the enzyme *Nhe*I. The purified fragments were individually cloned in the pED vector, digested with the enzymes *Sma*I (which generates blunt ends) and the *Xba*I which generates cohesive ends compatible with those obtained by *Nhe*I. The pED-endo and pED-$(his)_6$-met-endo constructs were transformed into *E. coli* DH5α and checked by restriction analysis of the inserted fragments.

### *Vector transfection, gene amplification, and endostatin expression*

The CHO mutant cell line, DXB11 (deficient in DHFR), described in Urlaub and Chasin [15] was maintained in minimal essential medium α-medium (α-MEM) containing 10 mg/L of ribonucleosides and deoxyribonucleosides (Cultilab, SP, Brazil) supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, 1.25 μg/mL fungisone, 2 mM L-glutamine, and 10% fetal bovine serum (FBS; Cultilab). Transfections of 20 μg of the purified vectors were carried out in plates containing $10^6$ cells using the calcium phosphate precipitation technique [16]. Cells were cultured for 24 h with medium containing 2% FBS and selection for DHFR-positive transformants was then applied by changing the medium to α-MEM without ribonucleosides and deoxyribonucleosides (Life Technologies, Grand Island, NY, USA), supplemented with 10% dialyzed FBS (Life Technologies), the antibiotics, and L-glutamine. After 2 weeks, some transformed DHFR-positive cells were transferred to a 6-well plate using plastic inoculating loops. Isolated clones were then subjected to increasing concentrations of methotrexate (MTX; 20, 80, 320, and 1280 nM) in the same medium for selection of clones containing amplified copies of the DHFR and endostatin genes. With each selection step, cells were cultivated for at least 14 days before increasing the MTX concentrations. To generate conditioned media for endostatin quantification, each transfected clone was seeded in three wells of a 24-well plate in α-MEM supplemented with antibiotics, L-glutamine, 10% dialyzed

FBS, and MTX, until semiconfluence was reached. For each clone, cells in one well were trypsinized and counted (cell number for time 0). The culture medium of the other two wells was then changed to the above medium without MTX, with and without FBS. Twenty-four hours later, conditioned medium was harvested and stored at −80 °C and the cell number for each well was determined by trypsin treatment and counting. The mean cell number between 0 and 24 h was then calculated for each condition.

*Gel electrophoresis and Western blot analysis*

Analysis of endostatin and $(his)_6$-met-endostatin in conditioned medium was performed by electrophoresis on 12% SDS–polyacrylamide gels under reducing conditions [17]. For SDS–PAGE used to compare endostatin expression by different clones/conditions, the volumes of medium applied to the gels were normalized to eliminate variations in cell densities. The gels were stained with Coomassie blue R-250 or the proteins were transferred by electrophoresis to a nitrocellulose membrane for immunoblotting. For Western blot, the membrane was probed for 10 h with a 1:150 dilution of the rabbit anti-mouse endostatin polyclonal antibody (Chemicon, Temecula, CA, USA) diluted in PBS containing 5% skim milk. Reactions were detected with secondary antibody conjugated to horseradish peroxidase (Amersham–Pharmacia Biotech, Buckinghamshire, UK) using enhanced chemiluminescence (Amersham–Pharmacia Biotech).

*Endothelial cell proliferation*

The ability of endostatin and $(his)_6$-met-endostatin to inhibit the proliferation of cow pulmonary artery endothelial cells (C-PAE) was tested by determination of their [$^3$H]thymidine incorporation. C-PAE cells, obtained from ATCC (CCL-209) (Manassas, VA), were maintained in Eagle's Minimal essential medium (EMEM) supplemented with 20% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. The cells were incubated in a humidified environment at 37 °C, in the presence of 5% $CO_2$. Incubations were performed in 96-well plates in a final volume of 200 μl/well of EMEM containing 2% FBS, with initial cell concentrations of $5 \times 10^3$ cells/well. After a 24-h incubation, the medium was replaced with fresh EMEM containing 2% FBS and 3 ng/mL of bFGF with or without endostatin. The cells were pulsed with 1 μCi [$^3$H]thymidine for 24 h, followed by harvesting of the cells. Cell-associated radioactivity was determined using a liquid scintillation counter.

*Determination of endostatin levels*

Endostatin levels in conditioned medium were determined by ELISA (Accucyte Mouse endostatin kit, Cytimmune Sciences, College Park, MD, USA) following the manufacturer's instructions.

*N-terminal sequencing*

The first seven amino acids of the N-terminals of the recombinant proteins were determined by solid-phase Edman degradation after electrophoresis and electroblotting of the band to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA).

## Results

*Construction of expression vectors*

The endostatin cDNA, encoding the amino acids MARRASVGTD$(H)_6$ plus the 184 residues corresponding to amino acids positions 1132–1315 of collagen XVIII, was cloned into the pSecTag2 vector containing sequences coding for the signal sequence of the murine Ig κ-chain V-J2-C. Mutagenesis was used to withdraw nucleotides to achieve in-frame coding of the signal sequence followed by endostatin or by $(his)_6$-met-endostatin. The sequences coding for signal peptide plus endostatin or the fusion protein cDNAs were cloned on the pED vector [18] under control of the SV40 enhancer

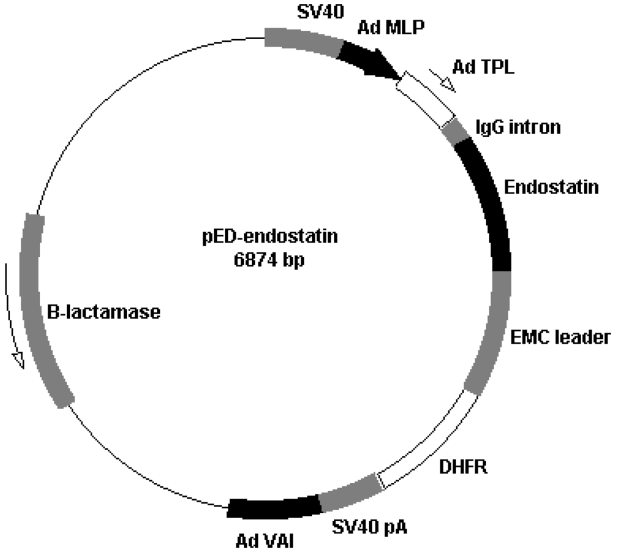


Fig. 1. Map of dicistronic vector pED-endo. The components are indicated as follows: SV40, fragment containing the SV40 origin of replication and enhancer element; Ad MLP, adenovirus major late promoter; Ad TPL, first two and 2/3 of the third leaders from adenovirus major late mRNAs; IgG intron, a hybrid intron composed of a 5′ splice site from the first leader of adenovirus major late mRNAs and a 3′ splice site from an immunoglobulin gene; endostatin, endostatin or $(his)_6$-met-endostatin cDNA; EMC-leader, the 5′ untranslated leader from the encephalomyocarditis virus; DHFR, a murine DHFR coding region; SV40 pA, the SV40 late polyadenylation signal; and AdVAI, the adenovirus VAI RNA gene, and β-lactamase, a selectable gene for propagation in *E. coli*.

element and adenovirus major late promoter. A diagram of the endostatin expression vector is presented in Fig. 1.

*Endostatin and (his)$_6$-met-endostatin expression*

Samples of conditioned medium produced by different clones of transfected CHO-DXB11 cells were analyzed by SDS–PAGE, after the last sequential amplification step (using 1280 nM MTX), for selection of clones expressing the highest levels of endostatin and (his)$_6$-met-endostatin (data not shown). Fig. 2 shows the SDS–PAGE of the selected endostatin-expressing clone which had been treated with increasing concentrations of MTX. While the 20 kDa band corresponding to endostatin cannot be seen in the columns corresponding to the non-transfected cells and to cells selected with 20 nM MTX, it can be seen when higher MTX concentrations were used: 80, 320, and 1280 nM. The increase in the levels of endostatin in the conditioned medium is probably due to amplification of the number of endostatin gene copies in the chromosomes of the transfected cells. Higher MTX levels were not applied since in general they do not lead to higher degrees of amplification [19].

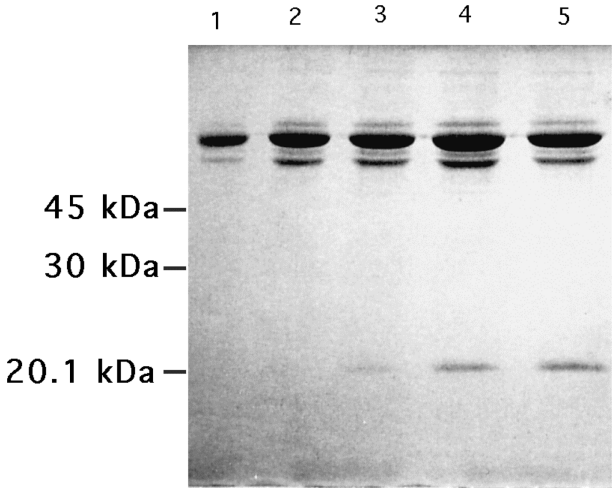


Fig. 2. Coomassie blue-stained SDS–PAGE (12.5%) analysis of endostatin obtained from 24 h-conditioned culture medium of transfected cells non-treated or treated with increasing MTX concentrations. Volumes applied to the gel (maximum of 20 μl) were normalized to eliminate variations in cell densities. Lane 1, non-selected cells. Lane 2, 20 nM MTX-selected cells. Lane 3, 80 nM MTX-selected cells. Lane 4, 320 nM MTX-selected cells. Lane 5, 1280 nM MTX-selected cells.

Analysis of endostatin and (his)$_6$-met-endostatin by SDS–PAGE (Fig. 3A) confirmed that the fusion protein has a slightly higher molecular weight than that of the authentic protein. Transfected cells cultivated without FBS produced (his)$_6$-met-endostatin and endostatin with low levels of contaminants, seen as faint bands of higher molecular weights. Western blot analysis of the recombinant proteins (Fig. 3B) further confirmed the identities of the recombinant proteins secreted by the selected clones.

The levels of endostatin and (his)$_6$-met-endostatin secreted by CHO cell lines transfected with the corresponding expression vectors and treated with 1280 nM MTX are presented in Table 1. The best-producing clones transfected with pED-(his)$_6$-met-endo reached endostatin secretion levels higher than pED-endo-transformed cells. Withdrawal of FBS from the medium

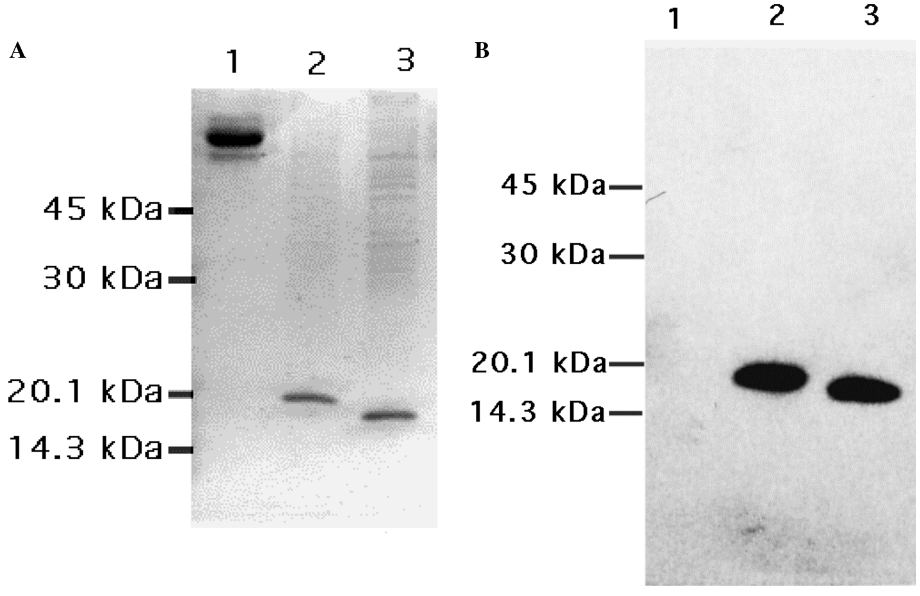


Fig. 3. (A) Coomassie blue-stained SDS–PAGE (12.5%) analysis of samples obtained from 24 h-conditioned culture medium. Lane 1, conditioned medium from non-selected cells. Lane 2, conditioned medium from a (his)$_6$-met-endostatin-expressing clone. Lane 3, conditioned medium from an endostatin-expressing clone. (B) Western blot analysis of conditioned medium of clones selected with 1280 nM MTX. Lane 1, non-transfected CHO cells. Lane 2, (his)$_6$-met-endostatin-expressing clone. Lane 3, endostatin-expressing clone.

Table 1
Endostatin and $(his)_6$-met-endostatin expression levels by transfected CHO cells

| | pED-endo (μg/$10^6$ cells) | pED-$(his)_6$-met-endo (μg/$10^6$ cells) |
|---|---|---|
| Medium with 10% FBS | 19.2 | 28.8 |
| Medium without FBS | 18.5 | 14.4 |
| Cells propagated for 1 month without MTX | 18.5 | * |

Results show Elisa determination of 24 h conditioned medium.
* Data not determined.

leads to a decrease of expression levels of endostatin and $(his)_6$-met-endostatin. The absence of MTX during 1 month propagation of the selected clone of pED-endo-transformed cells did not interfere with endostatin expression levels. For the cells cultivated 52 h in medium containing FBS, the levels of secreted native endostatin reached 78 μg/mL, while the one for secreted $(his)_6$-met-endostatin was 114 μg/mL.

*Biological activity*

The bioactivities of endostatin preparations were determined on the conditioned medium of endostatin-expressing CHO cells by its inhibitory activity on bFGF-stimulated C-PAE cell proliferation. The inhibitory effects of endostatin and tagged-endostatin, as determined by decreases in thymidine incorporation, are shown in Fig. 4. The inhibition range (0–91.4% of control) was seen with increasing concentrations of endostatin (0.8–114 μg/mL). The two recombinant proteins inhibited the replication of bovine endothelial cells in a dose-dependent fashion and showed similar inhibitory effects. To rule out the possibility that the inhibition of endothelial cell growth is caused by a secreted product of CHO cells other than endostatin, conditioned medium from CHO cells transfected with the pED vector without the endostatin gene and carrying amplified copies of DHFR gene was also tested. The inhibition of C-PAE cell growth was only 8%.

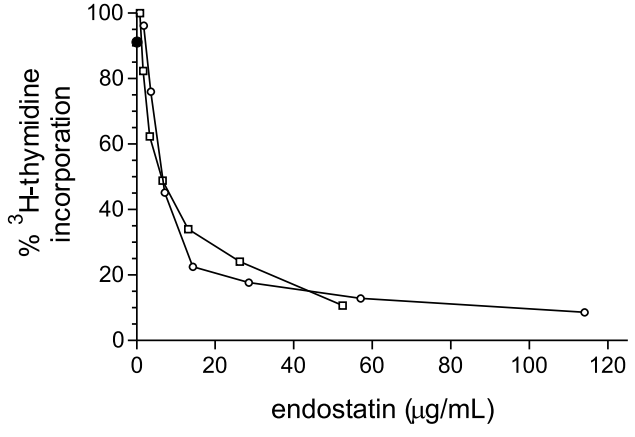


Fig. 4. Endothelial cell proliferation assay. Recombinant mouse endostatin (□) and $(his)_6$-met-endostatin (○) expressed in conditioned culture medium from CHO-transfected cells were tested for its ability to inhibit [$^3$H]thymidine incorporation in C-PAE cells. Conditioned medium from CHO cells transfected with the pED vector without endostatin gene (●) was used as a control.

*N-terminal sequencing*

The first N-terminal amino acids of endostatin determined via Edman degradation were HTHQDFQ, the same as those of the native protein. For $(His)_6$-met-endostatin, the sequenced N-terminal amino acids were HHHHHH(H)M. The additional histidine might be a residual peak from previous cycles or might be due to the difficulty of reading histidine residues on PVDF membranes. The results indicate that the signal peptide was correctly cleaved by the signal peptidase.

## Discussion

Transfection of the murine LM(TK-) cells with the pSecTag2-endo and pSecTag2-$(his)_6$-met-endo vectors, and selection of the clones resistant to hygromycin, resulted in clones expressing levels of up to 380 ng endostatin/$10^6$ cells/day and 55 ng $(his)_6$-met-endostatin/$10^6$ cells/day (data not shown). As these levels were very low, the former vectors were replaced with the pED vectors, using a CHO-DXB11 cell expression system to achieve higher endostatin expression levels.

The present paper describes the high-level synthesis of endostatin and of its fusion protein $(his)_6$-met-endostatin in CHO cells after their transfection with the dicistronic expression vectors, pED-endo and pEd-$(his)_6$-met-endo. A strategy of gene amplification using the selectable and amplifiable marker DHFR gene was followed. The utilization of the pED dicistronic expression vector design, containing an internal ribosomal binding site isolated from the EMC virus [18], was fundamental in achieving efficient gene amplification. An advantage of this system is that maintenance of selection conditions are not imprescindible. Transfected CHO cells selected in the presence of MTX for amplification of heterologous genes have proved to be stable upon propagation in the absence of MTX. The absence of this toxic substance during the production of a protein intended for therapeutic use against cancers is extremely important.

Clones were obtained which secreted 78 μg/mL endostatin and 114 μg/mL $(his)_6$-met-endostatin. Such levels are high and make it worthwhile to set up a purification process. The absence of serum in the medium in which the protein of interest is collected is desirable, since it facilitates downstream processing of the product and minimizes problems related to the use of this heterogeneous compound. Optimization of endostatin

production in a hollow fiber bioreactor could generate a highly concentrated recombinant protein, as already described for human prolactin expression using such expression system [20], with an increase of up to 30-fold in the concentration of the prolactin when compared to its production in culture dishes.

According to the data obtained by N-terminal sequencing, the signal peptide was correctly cleaved during secretion of the proteins, leading to the production of secreted forms of the heterologous endostatin and $(his)_6$-met-endostatin.

C-PAE cells were used for the assessment of the antiproliferative effect of endostatin on endothelial cells. This cell line was described as the one that gave the most reproducible response of inhibition of proliferation [12].

In addition to endostatin, $(his)_6$-met-endostatin was also produced because the histidine tag can be useful for the purification of the fusion protein by immobilized metal ion affinity chromatography. Another advantage of the presence of the six histidines is the potential use of anti-histidine antibodies for characterization and quantification of the tagged-protein. Such antibodies are commercially available at lower prices than specific anti-endostatin antibodies. As indicated by the C-PAE cell proliferation assay, the biological activity of the $(his)_6$-met-endostatin is comparable to that of the natural protein, suggesting that the presence of histidine tag in endostatin can be used, for example, as a marker for ''in vivo'' experiments.

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