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Purification and characterization of recombinant murine endostatin in *E. coli*

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Abbreviations: bFGF, basic fibroblast growth factor; CAM, cholioallantoic membrane; DMEM, Dulbeccos modified Eagles medium; BCS, bovine calf serum; FBS, fetal bovine serum; PBS, phosphate buffered saline; rmEndostatin, recombinant murine endostatin; LAL, Limulus Amebocyte Lysate; CD, circular dichroism

Abstract

Endostatin, a carboxyl-terminal fragment of collagen XVIII is known as an anti-angiogenic agent, that specifically inhibits the proliferation of endothelial cell and the growth of several primary tumor. We report here the purification and characterization of the recombinant murine endostatin (rmEndostatin) which was expressed in a prokaryotic expression system. This rmEndostatin has similar physiochemical properties of yeast-produced recombinant endostatin, and it also specifically inhibits the proliferation and migration of bovine capillary endothelial cells stimulated by basic fibroblast growth factor. The biological activity of rmEndostatin was also shown by its anti-angiogenic ability on the chorioallantoic membrane of chick embryo *in vivo*. In this article, we demonstrate the refolding and purification of rmEndostatin, expressed using *E. coli* system, to a biologically active and soluble form. In addition, these results confirm the activity of endostatin as a potent anti-angiogenic agent.

Keywords: recombinant endostatin, angiogenesis inhibition, prokaryotic expression system

Introduction

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is involved in various pathological conditions such as primary tumor growth, metastasis, diabetic retinopathy and wound healing (Colville-Nash and Willoughby, 1997). Several angiogenesis inhibitors, developed as anti-cancer drugs, are in pre-clinical and clinical trials (Zetter, 1998). Recently, angiostatin and endostatin, produced by a primary Lewis lung carcinoma and hemangioma respectively, were reported to be potent angiogenesis inhibitors (O'Reilly *et al*., 1994; O'Reilly *et al*., 1997).

Endostatin, a 20 kD carboxyl terminal fragment of collagen XVIII, is a specific inhibitor of the proliferation and migration of endothelial cells, and it also dramatically inhibits growth of the various primary tumors in mice (O'Reilly *et al*., 1997). Moreover, endostatin with repeat treatment on several tumors in mice was resulted in a persistent dormant state of the tumor (Boehm *et al*., 1997). The X-ray crystal structure of human and murine endostatin revealed that endostatin has a zinc binding site and a potent heparin binding site (Ding *et al*., 1998; Hohenester *et al*., 1998). In addition, this zinc binding of endostatin was reported to be essential for its anti-angiogenic activity and molecular stability (Boehm *et al*., 1998; Sasaki *et al*., 1998). The anti-angiogenic mechanism of endostatin is less clear and the generation of endostatin from collagen XVIII *in vivo* has not been understood in detail. However, recent experiment showed that endostatin caused G1 arrest and induced apoptosis in various endothelial cells, and these effects were not seen in non-endothelial cell types (Dhanabal *et al*., 1999a; Dhanabal *et al*., 1999b).

There are several article of the cloning and expression of recombinant endostatin to active soluble form using yeast (*Pichia Pastroris*) expression system (Dhanabal *et al*., 1999a; Dhanabal *et al*., 1999c). Recombinant endostatin expressed in a prokaryotic system was also reported to have angiostatic activity but difficult to refold to a soluble form and tend to precipitate out of solution (O'Reilly *et al*., 1997; Dhanabal *et al*., 1999c).

In our study, we report the refolding and purification of recombinant murine endostatin (rmEndostatin), expressed in prokaryotic system, to a biologically active and soluble form. The purified rmEndostatin expressed in *E. coli* was comparable to that obtained using Yeast-expression system not only in physiochemical properties but also in anti-angiogenic activities shown by *in vitro* and *in vivo* angiogenesis assay systems.

Materials and Methods

Construction of the recombinant plasmid expressing endostatin

The mc3b clone containing NC1 domain (Non collagenous C-terminal domain) of mouse α1 (XVIII) cDNA was a kind gift from Dr. B.R. Olsen. The sequence encoding endostatin was amplified by polymerase chain reaction (PCR) with Vent polymerase (New England Biolabs). The primer of 5'-end (ctaggatcccatatgCATACTCATCAGGACTTTCAG) contained *Nde* I site followed by annealing sequences of endostatin. The primer of 3'-end (atcggatccCTATTTGGAGAAAGAGGT) was designed to contain annealing sequence and a stop codon followed by *Bam* HI site. The PCR fragment was digested with *Nde* I and *Bam* HI and ligated into pET-15b vector linearized with *Nde* I and *Bam* HI and dephosphorylated by calf intestine alkaline phosphatase. The ligation mixture was first used to transform *E. coli* XL-1 Blue. After confirming the sequence, the recombinant plasmid was introduced into *E. coli* BL21 (DE3) for protein expression. The sequence of the constructs was confirmed by Chain termination DNA sequencing method using T7 sequenase version 2.0 DNA sequencing kit (Amersham).

Expression and purification of murine endostatin in *E. coli*

E. coli BL21 clones transformed with recombinant plasmid were grown in 3 L culture flask in LB media, and rmEndostatin was induced by 1 mM IPTG. Cells were harvested by centrifugation for 30 min at 5,000 g. Refolding and purification procedures of rmEndostatin were described briefly as follows; (i) cells were disrupted and the inclusion body (IB) was prepared by centrifugation and washed with deoxycholic acid. (ii) IB was then solubilized in Tris buffer (pH 8.6) containing 7 M guanidine-HCl and 50 mM β-mercaptoethanol, and refolding was performed with rapid dilution in Tris buffer (pH 8.6) containing 2.5 M urea and redox-coupling agents such as reduced and oxidized glutathione. For purification, the refolded rmEndostatin fraction was applied to SP-Sepharose resin (pharmacia), which was pre-equilibrated with 20 mM sodium phosphate buffer, pH 6.0. The resin was washed sufficiently with the equilibration buffer and then with the same buffer containing 0.5 M NaCl to remove nonspecific binding proteins. Refolded rmEndostatin fraction was eluted using the equilibration buffer containing 1 M NaCl. To remove His-tag portion of the rmEndostatin, thrombin cleavage was performed as previously described (Baubichon-Cortay *et al*., 1994). Thrombin was added to the purified protein (ratio of 1 : 250) in 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl and 2.5 mM $CaCl_2$ for 30 min at 25°C. The cleavage fragment was removed by using Superdex 75 HR 10/30 gel filtration resin (pharmacia).

Characterization of recombinant murine endostatin (rmEndostatin)

SDS-PAGE analysis was performed using precast 4~20% polyacrylamide gels according to the manufacturers instructions (Novex). Samples analyzed under reducing conditions were dissolved in the SDS sample buffer containing β-mercaptoethanol. For western blot analysis, anti-rmEndostatin serum was obtained from rabbit immunized with purified rmEndostatin. Reverse phase (RP) HPLC analysis was performed on C18 column (260× 4.6 mm, Vydac). A Gilson HPLC system was used with 5~95% acetonitrile gradients for 45 min with a flow rate of 1 ml/min. Circular dichroism (CD) spectra of rmEndostatin were measured on a JASCO J-715 spectropolarimeter using 1.0 cm path length cell at room temperature. Proteins were dissolved in 50 mM sodium phosphate, pH 7.4 at the final concentration of 0.1~0.15 mg/ml, and filtered with 0.45 mm pore size filter unit before use. Protein concentration was measured using Lowry method.

Cell culture

Bovine capillary endothelial (BCE) cells were kindly provided by Dr. Lee H-T in Yonsei university (Lee *et al*., 1998). Cell culture was performed as previously described (Folkman *et al*., 1979). BCE cells were maintained in DMEM containing 10% heat-inactivated BCS (bovine calf serum, GibcoBRL), 3 ng/ml bFGF (UBI), and 1% antibiotics (GibcoBRL).

Lewis lung carcinoma cell and NIH 3T3 cell were purchased from American Type Culture Collection. Lewis lung carcinoma cells were maintained in DMEM containing 10% heat-inactivated FBS (fetal bovine serum, GibcoBRL) and 1% antibiotics (GibcoBRL), and NIH 3T3 cells were maintained in the same media as that for BCE cells.

Cell proliferation assay

BCE proliferation assays were performed as previously described (O'Reilly *et al*., 1994). Briefly, cells were washed with PBS and were dispersed in a 0.05% of trypsin solution. A cell suspension was made with culture medium and the concentration was adjusted to 25,000 cells/ml after hemocytometer count. Cells were plated onto gelatinized 24 well culture plates (0.5 ml/well) and were incubated (37°C in 10% CO_2) for 24 h. After replacement of the media with 0.25 ml of DMEM containing 5% BCS and 1% antibiotics, test samples were applied. After 30 min of incubation, media and bFGF were added to each well to obtain a final volume of 0.5 ml of DMEM containing 5% BCS, 1% antibiotics and 1 ng/ml bFGF. The plates were incubated for 72 h, and then cells were dispersed in trypsin, resuspended in PBS and counted with hemocytometer. Each sample was tested in triplicate. Non-endothelial cell proliferation assays were also performed in the same condition as of the BCE cells.

Cell migration assay

Endothelial cell migration assay was accomplished with

a modified Boyden chamber based assay (Ji *et al*., 1998). Transwell polycarbonate membrane with 8-μm pore size (Costar) was coated with 0.1% gelatin (Gibco-BRL). After BCE cells were trypsinized and washed, cells were resuspended in DMEM containing 10% bovine calf serum and 10 ng/ml bFGF at a concentration of 75,000 cells/ml, and incubated at 37°C for 30 min. During cell incubation, rmEndostatin with 5 ng/ml bFGF was loaded into the lower chamber with media. The gelatin-coated membrane filter was placed on top of the lower chamber and the top chamber was then attached. After cell incubation, BCE cells were loaded into the top chamber and incubated at 37°C for 16 h. The chemotaxis chamber was then dismantled and the filter membrane was removed. The non-migrated cells were scraped from the upper surface of the membrane with cotton swabs three times. After rinsing with PBS, the membrane was fixed with 10% buffered formalin for 45 min and then stained with hematoxylin Gill No. 2 (Sigma) overnight. The number of migrated cells was counted on the hemocytometer and each sample was tested in quadruplicate.

Chorioallantoic membrane (CAM) assay

To determine anti-angiogenic activity *in vivo*, CAM assay was performed as previously described (Nguyen *et al*., 1994). Fertilized 3-day old eggs were incubated at 37°C, 90% of humidity. After 2 day of incubation, 4~5 ml of ovalbumin was extracted in the bottom of the eggs using syringe. After 3 day of incubation, fertilized eggs were made with window in the top of the eggs. After 4.5 day of incubation, thermonox (Nunc) coverslips (1/4 pieces) with test samples were applied on the CAM of individual embryos. After 7 day of incubation, embryos and CAMs were observed by means of stereomicroscope, and intralipose (white fat emulsion solution) was injected into the CAM using syringe for distinct discrimination of the blood vessel formation.

Results

Expression and purification of rmEndostatin

The recombinant murine endostatin (rmEndostatin) was expressed as inclusion body, and was refolded as a soluble form in the presence of chaotrophic agent (urea and guanidine-HCl) and redox-coupling reagents such as glutathione (Figure 1A; lane 1 and 2). Refolded rmEndostatin was purified to a single peak using SP-sepharose and Superdex 75 column. The rmEndostatin was eluted at high salt fraction on SP-Sepharose column because endostatin is a very basic protein (calculated pI; 9.2). The rmEndostatin can be also purified using Heparin-Sepharose column due to its heparin-binding affinity (Dhanabal *et al*., 1999c). N-terminal His-tag region of rmEndostatin was removed by thrombin cleavage and gel filtration chromatography. The purified rmEndostatin was confirmed with N-terminal sequence analysis.

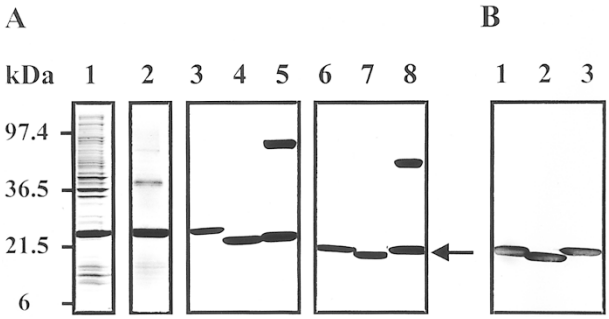

Figure 1. Expression and purification of the rmEndostatin in *E. coli.* A. SDS-PAGE analysis of purified rmEndostatin. Samples were loaded onto 4~20% gradient gel followed by staining with Coomassie-Blue; lane 1: Cell lysate with 1 mM IPTG, lane 2: Isolated inclusion body, lane 3 and 6: rmEndostatin purified with SP-Sepharose chromatography, lane 4 and 7: rmEndostatin purified with Superdex 75 after thrombin cleavage, lane 5 and 8: Yeast-produced murine endostatin (Calbiochem). The upper band indicates albumin as stabilizer. Lane 6, 7 and 8 were performed under non-reducing conditions and other lanes were performed under reducing conditions, respectively. The arrow indicates purified rmEndostatin and Yeast-produced endostatin. B. Western blot analysis of purified rmEndostatin; lane 1: rmEndostatin purified with SP-Sepharose chromatography, lane 2: rmEndostatin purified with Superdex 75 after thrombin cleavage, lane 3: Yeast-produced murine endostatin. All lanes were performed under non-reducing conditions.

We obtained about 16 mg of soluble purified rmEndostatin from 1 L of flask culture broth, which would be more than 10% of purification yield. Compared to the reported recombinant endostatin expressed in *E. coli*, where most of the endostatin was precipitated out of solution, we were able to successfully refold and purify the rmEndostatin expressed in prokaryotic expression system to a soluble forms with a high purification yield.

Characterization of rmEndostatin

The purified rmEndostatin has an apparent molecular weight of 20 kD on the SDS-PAGE analysis under reducing conditions, and yeast-produced murine endostatin has also a similar molecular weight (Figure 1A). Both of the recombinant endostatin has a slight difference in mobility on the gel between reducing and non-reducing conditions as shown Figure 1A. The slight shift to a slower mobility after reduction is due to opening of the internal disulfide bonds. And these results were coincident with the previous report on the recombinant endostatin expressed in human embryonic kidney cells (Sasaki *et al.,* 1998). In case of rmEndostatin where N-term. His-tag region was removed, the band was shifted down on the gel.

Western blot analysis demonstrated that rmEndostatin and yeast-produced murine endostatin were also clearly recognized by an anti-rmEndostatin rabbit serum (Figure 1B). Structural aspect of rmEndostatin was confirmed by circular dichroism (CD) analysis. CD spectra of rmEndostatin were measured with a characteristic minimum at

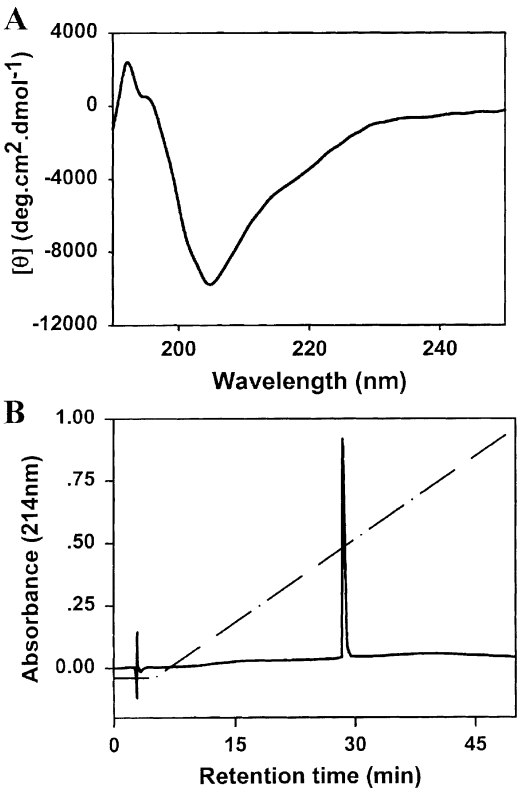

Figure 2. Characterization of purified rmEndostatin. A. CD spectra of rmEndostatin: CD spectra of rmEndostatin in 50 mM sodium phosphate, pH 7.4, at room temperature. B. RP-HPLC analysis of rmEndostatin: purified rmEndostatin was injected on protein C18 reverse phase chromatography (Vydac) with a linear acetonitrile 5~95% gradients for 45 min, 1 ml/min of flow rate. Solution A: 0.1% (v/v) TFA (trifluoroacetic acid) in distilled water. Solution B: 0.085% (v/v) TFA in acetonitrile. The dash and dotted line indicates the linear gradient of acetonitrile.

205 nm as shown in Figure 2A. Secondary structure estimates with Chang's equation indicated the rmEndostatin to have about 10.9% of α-helix and mostly β-sheet structure. These data were similar with the pervious report on recombinant endostatin (Sasaki *et al.,* 1998). In the analysis of RP-HPLC, purified rmEndostatin showed a single peak as shown in Figure 2B, and there were no polymers or degraded fragments in the fraction of purified rmEndostatin. These results were confirmed with SDS-PAGE analysis (Figure 1A)

All the above results indicate that the rmEndostain, which was expressed and purified in a prokaryotic expression system, has similar physiochemical properties of yeast-produced form.

BCE cell assay

To evaluate the function of rmEndostatin on endothelial cells, proliferation and migration assay of BCE cells were performed as previously described (O'Reilly *et al.,* 1994; Ji *et al.,* 1998). rmEndostatin inhibited bFGF-induced proliferation of BCE cells *in vitro* in a dose-dependent manner (Figure 3A). The ED_{50} of rmEndostatin in proliferation of BCE cells was approximately 0.2-0.3 μg/ml and it was comparable with that of the yeast-produced endostatin. There was no difference in anti-angiogenic activity whether N-terminal His-tag region was intact or not (data not shown). In addition, there were no anti-proliferative effects on non-endothelial cells such as tumor cell (Lewis lung carcinoma) and normal cell (NIH 3T3 cell) as shown in Figure 3B and C. And these results were in consistent with previous results (O'Reilly *et al.,* 1997).

To exclude influence of endotoxin on BCE cells, the endotoxin of the purified rmEndostatin fraction was removed using ion-exchange column (DEAE-Sepharose and SP-Sepharose, Pharmacia). The endotoxin of rmEndostatin was determined to be less than 0.001EU/well by LAL (Limulus Amebocyte Lysate) test, and there was no evidence of BCE cell detachment from the assay plates even at the highest concentration of rmEndostatin treated before cell counting.

rmEndostatin also inhibited migration of bFGF-induced

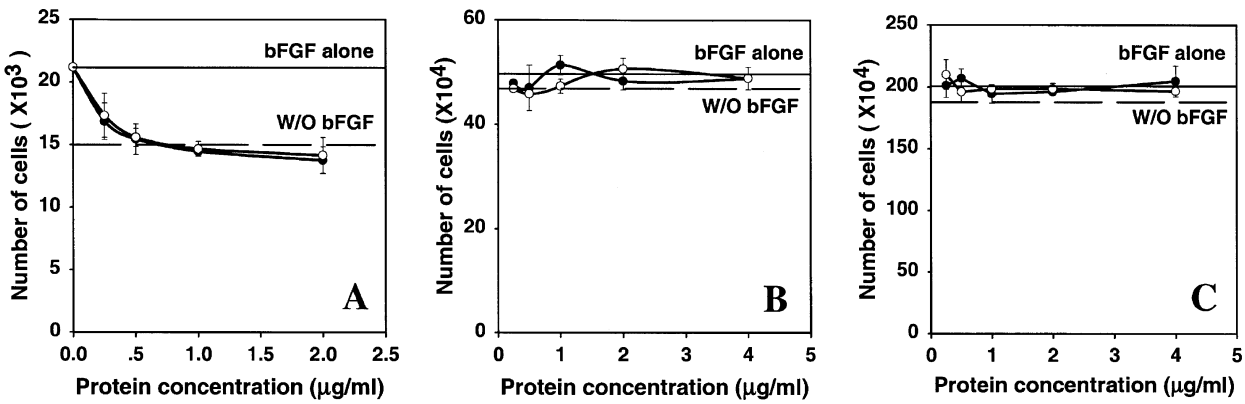

Figure 3. Cell proliferation assay *in vitro*. A. Purified rmEndostatin (○) and Yeast-produced murine endostatin (●) inhibit BCE cell proliferation in a dose-dependent manner with a half-maximal dose, less than 0.3 μg/ml. Solid line indicates the positive control with bFGF (1 ng/ml) alone, and dashed line indicates negative control. B and C. Purified rmEndostatin and Yeast-produced murine endostatin have no effect on the proliferation of non-endothelial cells such as Lewis lung carcinoma cell (B) and NIH 3T3 cell (C), respectively

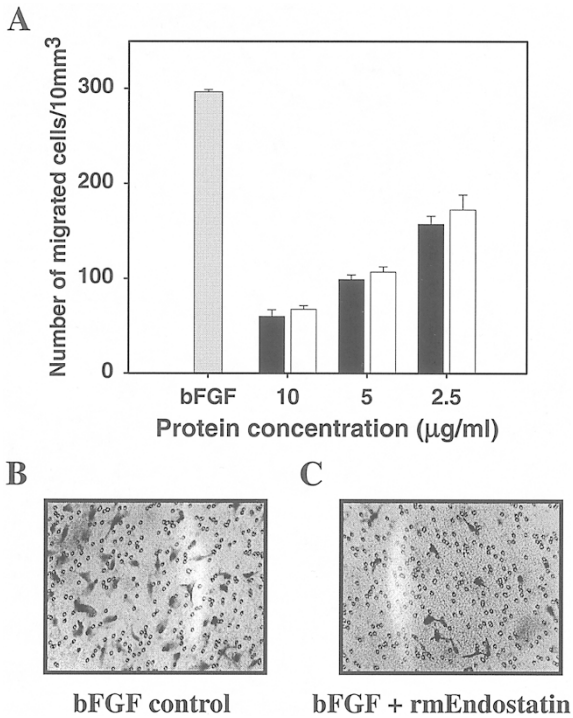

Figure 4. Migration assay with purified rmEndostatin. A. Purified rmEndostatin (□) and yeast-produced murine endostatin (■) inhibit BCE cell migration on gelatin-coated transwell membrane (8 μm) in a dose-dependent manner. B and C. Migrated BCE cells on the transwell membrane were stained with hematoxylin Gill No. 2 in the absence or presence of endostatin (10 μg/ml), respectively

BCE cells (Figure 4). The migration of BCE cells were inhibited by rmEndostatin in a dose-dependent manner (Figure 4A), and migration inhibition of BCE cells can be clearly seen through microscopy (Figure 4B and C).

CAM assay

To determine whether rmEndostatin has an anti-angiogenic activity *in vivo*, anti-angiogenic activity of rmEndostatin was determined using chorioallantoic membrane (CAM) assay. rmEndostatin was able to potently inhibit the neovacularization in chick embryo in a dose-dependent manner with ED_{50} of 0.5 μg/embryo (Figure 5). When embryo was treated with more than 10 mg of rmEndostatin, new blood vessel formation in chik embryo CAM was completely inhibited. Moreover, some chick embryo was not able to grow up to next developmental stage and died after all.

Discussion

Recently, anti-angiogenic agents are being watched as useful cancer therapy (Harris, 1998). Among these anti-angiogenic agents, endostatin was recognized as one of the most powerful anti-cancer agents with fewer side effects. To develop endostatin for cancer therapy, it must

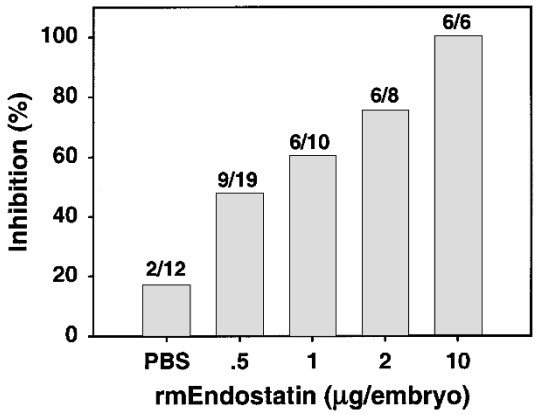

Figure 5. Anti-angiogenic effect of purified rmEndostatin on the chick CAM *in vivo*. The number of anti-angiogenic zone and the number of CAMs for each group are indicated above each bar. rmEndostatin inhibits angiogenesis on the CAMs in a dose-dependent manner. Inhibition (%) of treated samples was measured with number of anti-angiogenic CAMs by total number of treated CAMs.

be produced in large quantity. Several expression systems of endostatin were reported (O'Reilly *et al.,* 1997; Sasaki *et al.,* 1998; Dhanabal *et al.,* 1999c). One was eukaryotic system and the other was prokaryotic system. The recombinant endostatin, produced by eukaryotic expression systems (baculovirus and yeast), showed not only anti-angiogenic activities *in vitro* and *in vivo* assay systems but also anti-cancer activties in various tumor grown animal models. However, there are some disadvantages of using the eukaryotic expression systems for large-scale production such as low production yield and high production cost. In this point, prokaryotic expression system is more useful and economical than eukaryotic system. There are some reports of endo-statin expressed in *E. coli*; however, the *E. coli*-derived recombinant endostatin was tend to precipitate and its final yield was reported to be less than 1%. This low yield of recombinant endostatin expressed in *E. coli* was probably due to the refolding process of its protein. Generally, in the refolding process of recombinant protein, protein concentration and the pH of refolding buffer are very important factor to determine the yield of purification and the efficacy of refolding (Fischer *et al.,* 1993).

In this study, we demonstrate the refolding and purification of the recombinant murine endostatin, expressed in prokaryotic system, to a soluble and biologically active form. Refolding of the solubilized inclusion body (IB) was accomplished by rapid dilution method in slightly basic refolding buffer containing 2.5 M urea and redox-coupling agent with 1 : 100 of dilution factor. At this dilution factor, the protein concentration of this refolding fraction became less than 0.1 mg/ml. Refolded and soluble rmEndostatin was purified with a single peak on SP-sepharose column at high salt elution step. SP-Sepharose column was useful than heparin-affinity column due to chemical stability to high concentration of urea, therefore we use SP-Sepharose

instead of heparin-Sepharose to purify recombinant endostatin. The final yield of rmEndostatin was about 10% and it can be improved by adding high molecular weight protein stabilizer or basic amino acids such as polyethylene glycol or arginine, respectively (Rudolph and Lilie, 1996).

To determine if the *E. coli*-derived rmEndostatin was folded as the native endostatin, the purified rmEndostatin was characterized. The *E. coli*-derived rmEndostatin has a similar molecular weight and heparin binding ability as the yeast-produced endostatin. Moreover, poly-clonal antibody raised by rmEndostatin has immuno-chemical cross-reactivity with yeast-produced murine endostatin. Conformation analysis by CD also indicates rmEndostatin to have similar molecular structure of native or yeast-produced murine endostatin. In addition, the rmEndostatin has biological anti-angiogenic activities (determined by *in vitro* and *in vivo* angiogenesis assay) comparable to yeast-produced murine endostatin. These results show the possibility to produce recombinant active endostatin in large scale and high yield from *E. coli*. With this system, it would be easier to make quantity of endostatin to do further studies on mechanism as well as efficacy of the protein.

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