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Biochemical and Biophysical Research Communications 345 (2006) 1398–1404
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Soluble multimer of recombinant endostatin expressed in *E. coli* has anti-angiogenesis activity

Dong-mei Wei [a,b], Yan Gao [d], Xiang-rong Cao [b], Nian-chun Zhu [a], Jian-fu Liang [c], Wei-ping Xie [c], Ming-ying Zhen [c], Min-sheng Zhu [a,*]

[a] *Model Animal Research Center, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210061, PR China*
[b] *Life Science College of Nanjing Normal University, Nanjing 210097, PR China*
[c] *Star Lake Bioscience Co. Inc., Zhaoqing Guangdong, 526040, PR China*
[d] *School of Basic Medical Sciences, Zhengzhou University, Zhengzhou 450052, PR China*

Received 4 May 2006
Available online 15 May 2006

Abstract

The bioactivity, refolding, and multimer formation of endostatin, particularly of recombinant endostatin produced from bacteria, are proved challenging for clinical application. In order to determine the biological activity of recombinant endostatin multimer, first, we expressed endostatin in *Escherichia coli* and purified it with ion-exchange chromatography. The purified active protein could elicit multimer formation spontaneously, but still has comparable activity. Aim to determine the anti-angiogenic activity of multimer endostatin, by use of RP-HPLC, we then successfully separated endostatin monomer and multimer for subjecting to anti-angiogenesis assay. The results from CAM (chorioallantoic membrane) inhibition assay showed that both monomer and multimer suppressed CAM vascularization significantly. At the dosage of 0.8 μg, inhibition rates of multimeric and monomeric proteins were about 58% and 38%, respectively. Multimeric endostatin exerted a higher activity than monomeric endostatin ($p < 0.05$). However, when the protein dosage is less than 0.4 μg/ml, there is no significance between their inhibition rates ($p > 0.05$), although both of them show a high inhibition effect in contrast to control. The results from HUVEC proliferation assay also showed similar effects at dosages of 0.6 and 1.6 μg/ml, multimer exerted a higher activity on inhibition of HUVEC proliferation comparing with monomer ($p < 0.05$). In conclusion, our results suggest that endostatin multimer has a comparable or higher bioactivity and multimerization will not affect its bioactivity, implying that endostatin activity is insensitive to structure conformation contributed by disulfide bonds.

Keywords: Endostatin; Multimer; Ion-exchange chromatography; RP-HPLC; Anti-angiogenesis

Endostatin is a 184-aa C-terminal proteolytic fragment of collagen XVIII extracted from mice endothelial tumor cell's culture medium and specifically inhibits tumor angiogenesis [1]. Results from animal studies demonstrated that recombinant endostatin strongly inhibited the growth of a variety of murine and xenotransplanted human tumors [2,3]. The recombinant endostatins have been produced both from *Escherichia coli* and yeast successfully [4] and have been undergoing clinical investigation [5]. Due to the relatively low yield and high cost of yeast expression system, the bacterial expression system is an alternative method for endostatin production. However, the refolding and purification of bioactive endostatin from *E. coli* have been proved challenging [6,7]. The reasons are included: (1) After removing the denaturing detergent (e.g., urea) during refolding processes, recombinant protein is very easy to pellet under physiological condition, despite the insoluble protein has an anti-angiogenesis activity in vivo; (2) the soluble protein prepared by refolding and purification may form multimer gradually. Generally, multimer formation is considered as a result of misfolding or incomplete refolding and such multimerized proteins usually were

* Corresponding author. Fax: +86 2558641500.
E-mail address: zms@nicemice.cn (M. Zhu).

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doi:10.1016/j.bbrc.2006.05.031

inactive or partially active. Therefore, as we know, high concentration of multimer in the protein product is restricted to clinical application. Besides the reason that it will easily cause protein aggregation and precipitation (even in invisible form), loss of bioactivity for the multimeric protein is another important reason. However, in our previous experiments, the soluble endostatin containing multimer still has a comparable anti-angiogenesis activity [our unpublished data]. Thus, the question is whether the endostatin multimer also has antiangiogenic activity. To address this question, we purified endostatin monomer and multimer individually with RP-HPLC and determined its bioactivity through chorioallantoic membrane (CAM) inhibition assay and endothelial cell proliferation assay. These results showed a comparable or even higher bioactivity of multimer in contrast to monomer endostatin, implying that endostatin activity is insensitive to its structure conformation contributed by disulfide bonds.

Materials and methods

Materials. Human umbilical vein endothelial cells (HUVEC), recombinant plasmid pET-28a/endostatin, and BL-21 were kept in our laboratory, and mouse polyclonal antibody of human endostatin was prepared in our laboratory. Anti-human CD31 monoclonal antibody, Goat anti-mouse IgG-HRP, and Goat anti-mouse IgG-FITC were purchased from Sigma. DEAE–Sepharose FF and CM–Sepharose FF were purchased from Pharmacia. The apparatus model of RP-HPLC is Waters2996-PDA600E (Waters).

Expression of endostatin. The recombinant plasmid pET-28a/endostatin was transformed into competent BL-21 cells by regular methods. At the mid-log growth phase, 1 mM IPTG was added to induce endostatin expression. The induced cells were harvested by centrifugation at 8000*g* for 10 min. Cell pellet was re-suspended in lysis buffer (20 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole, and 0.1% lysozyme, pH 7.9) and ultrasonicated on ice. The pellet for inclusion body purification was prepared by centrifugation at 12,000*g* for 20 min.

Purification of endostatin. All subsequent steps were performed at 4 °C unless otherwise indicated. The endostatin inclusion body was washed with buffer 1 (3 M urea/50 mM Tris–HCl, pH 8.3/1 mM EDTA), centrifuged at 4500*g* for 20 min and dissolved in buffer 2 (8 M urea/20 mM Tris–HCl, pH 8.3/1 mM EDTA/5 mM β-mercaptoethanol (2-ME)). The solution was stirred for 2 h and centrifuged at 9000*g* for 15 min. The supernatant was loaded onto a DEAE–Sepharose FF column equilibrated with 20 mM Tris–HCl, pH 8.5. The protein in flow-through fraction was applied to CM–Sepharose FF column after adjusting pH value to 5.0. Protein samples eluted by 150 mM NaCl were collected and dialyzed against buffer 3 (1 M NaCl/20 mM Tris–HCl, pH 8.3) overnight. The precipitation was re-dissolved in buffer 4 (8 M urea/50 mM Tris–HCl, pH 5.0) and then dialyzed against 2000 ml distilled water containing 400 μl acetic acid. The protein solution was centrifuged followed by filtration with 0.22 μm filter and the soluble endostatin in supernatant fraction was obtained. The purity was always higher than 98%.

Separation of monomer and multimer. The purified endostatin was filtered through 0.22 μm filter again prior to subjecting to RP-HPLC separation. The HPLC system consisted of a Waters 600E separation module (Waters, Milford, MA) and a Model 2996 photodiode array detector. The chromatographic data were collected and analyzed using Empower Chromatography Manager (Version 4.0, Waters). Separation was achieved at ambient temperature with a Waters Symmetry™ C4 column. The column dimensions were 4.6 × 250 mm with a stationary phase of resin particles in size of 5 μm in diameter. The mobile phase consisted of solvent A and solvent B. Solvent A was water containing 0.1% TFA (v/v) and solvent B was acetonitrile plus 0.1% TFA (v/v). Proteins were separated at a flow rate of 1 ml/min by a linear gradient program (minutes; % solvent A; % solvent B): (0; 100; 0), (15; 60; 40), and (40; 0; 100). Each run required 40 min and 100 μl of injection volume. All solvents were of HPLC grade. Repeat sample injections and manual collections were performed. The collection samples were determined by SDS–PAGE and Western blot assay. The primary antibody was mouse polyclonal antibody against human endostatin and the second antibody was goat anti-mouse IgG-HRP.

Chick chorioallantoic membrane assay. To determine anti-angiogenic activity in vivo, CAM assay was performed according to the method [8]. All procedures were carried out in a laminar flow hood under sterile conditions. The fertilized 6-day eggs were incubated at 90% of humidity and 37 °C. A window was made on the top of each egg. The samples including Endostatin and appropriate buffer controls were spotted onto sterilized Whatman filter paper disks and applied to the surfaces of the growing CAMs above the dense subectodermal plexus. Forty-eight hours after implantation with filter paper disk, embryos and vascularization degree were quantified by counting the number of branch of blood vessel. Average values for five to eight embryos per treatment were recorded. The statistical significance was tested by Student's *t* test.

Endothelial cell proliferation assay. HUVEC were cultured as previously described [9]. Cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotics. Cell proliferation assays were performed as the following: briefly, human umbilical vein was washed with PBS and dispersed in trypsin solution. Cell suspension was made with culture medium and the concentration was adjusted to 3000 cells/100 μl. Cells were plated onto 96-well culture plates (0.1 ml/well) and incubated for 24 h. After replacement of the media with 100 μl DMEM containing 5% FBS, 1% antibiotics, and 5 ng/ml bFGF, different dosages of endostatin were added into each well. The plates were further incubated for 48 h, and then the cells were stained with 0.5% crystal violet and the $OD_{592\ nm}$ readings were expressed as ratio of survival cells. NIH3T3 cells were used as a control of proliferation assay. In order to verify the identity of HUVEC, the cells within four passages were cultured on coverslips and stained with anti-CD31 monoclonal antibody (Sigma). FITC-labeled anti-mouse antibody was used as secondary antibody and fluorescent marker was examined under confocal microscope.

Results

Expression and purification of endostatin

After transformation with endostatin-expressible plasmid and induction with IPTG, BL-21 host cells expressed recombinant protein efficiently. The expression rate of recombinant endostatin is about 30–40% of total bacterial protein (Fig. 1). Most of endostatin protein existed in the form of inclusion body. After purification with

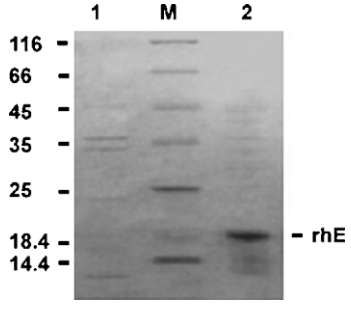

Fig. 1. SDS–PAGE analysis of the expression of recombinant endostatin. The middle molecular weight marker was loaded in lane M and sample of recombinant human endostatin was loaded in lane 2; BL-21 host cell lysate was used as a control (lane 1); rhE indicates recombinant endostatin.