·Basic Research·

Preparation of endostatin protein and the measurement of its biologic activity

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Abstract

• AIM: To investigate the preparation of endostatin protein and its biologic activity on vascular endothelial cell.

• METHODS: pBlast-hEndostatin and pBlast-Mcs were identified by digesting with Nhe Ⅰ and Sal Ⅰ, by PCR reaction, by sequencing, and by alignments of PCR products with gene bank using NCBIBLAST software. The identified pBlast-hEndostatin as well as pBlast-Mcs were then purified with QIAGEN Endofree plasmid maxi kit. The purified plasmids transfected human fibroblasts. The expression of endostatin was detected by RT-PCR,Western blot and immunohistochemistry. The endostatin protein produced by transfected fibroblasts was purified by ultrafiltration and affinity chromatography. The inhibitory action of endostatin on human umbilical vein endothelium was measured by MTT assay.

• RESULTS: pBlast-hEndostatin was found to contain human endostatin gene. Endostatin protein was produced by transfected fibroblasts. The inhibitory ratio of 2.5,5,10,20, 40,80mg/L endostatin on human umbilical vein endothelium for 48 hours were 8.5%,13.1%,27.7%,38.1%,56.7%,63.8% respectively. IC50 value was 34.5mg/L. No inhibitive action was found on fibroblasts.

• CONCLUSION: Endostatin protein can be produced by the transfected fibroblasts. The produced endostatin has inhibitory action on human umbilical vein endothelium and has no inhibitive action on fibroblasts.

• KEYWORDS:endostatin;protein preparation;biologic activity

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INTRODUCTION

Development of abnormal new vessels have close correlation with development of many oculopathies, such as age-related macular degeneration, diabetic retinopathy, neovascular glaucoma, and cornea neovascularization which often lead to decreased vision or blindness. In addition, neovascularization is also closely related to the occurrence, growth and metastasis of ocular tumors. In order to prevent and treat those diseases, investigators have been vigorously searching for inhibitors against neovascularization. In these inhibitors endostatin was found to be able to specifically inhibit vascular endothelial cell proliferation, and is recognized as one of the most potent antiangiogenesis agents at present. This factor also has the benefits of no toxicity and no drug resistance after a long time use. It could be an ideal antiangiogenic biological agent. In this paper we investigated the preparation of endostatin protein and its biologic activity on vascular endothelial cells. The results may provide additional information for the treatment of oculopathies related to neovascularization.

MATERIALS AND METHODS

Materials Plasmid: pBlast-hEndostatin and pBlast-Mcs (Invivogen, USA); QIAGEN Endofree plasmid maxi kit (Qiagen); Blasticidin (Invivogen); Enzyme Nhe Ⅰ and Sal Ⅰ (MBI); Taq DNA polymerase (Boehringer mannheim); PMD18-T Vector (Dalian biology engineering limited company); lipofectamine2000 (Gibco); 2.5 ×1mL Hi-trap Heparin column (Pharmacia); Human umbilical vein endothelial cells (Wuhan University Culture Collection Center); MTT (sigma); M199 (Gibco); HEPES (Gibco); Altrafree-15 Centrifugal Filter Device (millipore); Rneasy Mini Kit (Qiagen); RT-PCR kit (Epicentre Technologies); Western blot Detection kit (Cell Signaling Technology); RT-PCR primers 5'end: GTG CCC ATC GTC AAC CTC A; 3'end: TCC GCC ACG TCT CAC AGT AG.

Methods

Identification, proliferation and purification of the plasmids of pBlast-hEndostatin and pBlast-Mcs ① pBlast-hEndostatin and pBlast-Mcs were grown under blasticidin selection in host strain DH5α ; ② purified plasmids by alkaline lysis; ③ digested pBlast-hEndostatin with Nhe I and Sal I, and digested pBlast-Mcs with Sal I; ④ PCR was carried out for 30 cycles using standard conditions. The primers used were: AA GAT ATC ATG CAC AGC CAC CGC GAC TTC and GA GCT AGC CTA CTT GGA GGC AGT CAT G based on pBlast-hEndostatin sequence; ⑤ sequenced PCR product by Dalian biology engineering company; ⑥ purified plasmids using QIAGEN Endofree Plasmid Maxi kit.

Human fibroblast culture Digestion method was used: The fibrous connective tissue of bulbar conjunctiva and orbitalis was cut into small pieces and digested by 2.5g/L trypsin for about 30 minutes. Then the cells were cultured conventionally. The cultured cells were identified by HE and immunohistochemistry staining.

Transfection of pBlast-hEndostatin and pBlast-Mcs and screening of the transfected human fibroblast cells The transfection was performed according to Lipofectamine 2000 transfection instructions: 1×10^6 fibroblast cells were subcultured in cultivation plates at 37℃, 50mL/L CO_2 incubation for 24 hours until 80% cell fusion was achieved. pBlast-hEndostatin, pBlast-Mcs and lipidosome were added into the plates respectively for 5 hours. Then the transfection solution was drawn off and completely replaced by culture solution that contained 100mL/L fetal bovine serum. After cultivated in 37℃ 50mL/LCO_2 incubation for 48 hours, the cells were screened by Blasticidin at concentration of 2mg/L and macrocultured.

Identification of endostatin produced by transfected fibroblast ① The transfected fibroblasts were trypsinized and collected. The cell's total RNA was extracted by RNA extraction kit. RT-PCR reaction was performed according to RT-PCR kit instruction. PCR amplification reaction: thermal denaturation at 95℃, 30 seconds; annealing at 52℃, 30 seconds; extending at 72℃, 60 seconds; 35 circulations, the last circulation at 72℃ extending for 5 minutes. ②After the transfected fibroblasts were disrupted by cell disruption solution and centrifugated, the endostatin in the supernatant fluid was detected with Western blot method by the kit instruction. ③After the transfected fibroblast was fixed with acetone, immunohistochemistry reaction was conducted by the kit instruction to detect the endostatin protein.

Purification of endostatin protein ① The cell disruption solution of transfected fibroblasts and supernatant of the cell culture was centrifugally filtrated through millipore filtrator of 50ku diameter, and the effluent was collected. ② The above effluent was centrifugally filtrated though millipore filtrator of 5ku diameter, and the concentrated solution was collected. ③ The above solution was diluted with binding buffer solution. ④ The above solution was put into the heparin affinity column balanced with binding buffer solution (0.5mL/min). ⑤ Endostatin protein was eluted with elution buffer solution of different concentration. ⑥ The effluent was collected and A 280 and A 260 values were measured with ultraviolet spectrophotometer. ⑦ Endostatin protein was detected with SDS-PAGE, Western blot methods. ⑧ The above solution was dialysed with 0.1mol/L PBS three times at 24 hours at 4℃. ⑨ The purified protein was vacuum dehydrated and conserved in -70℃ freezer.

Evaluation of biologic activity of endostatin protein After human umbilical vein endothelial cells and fibroblasts of exponential phase of growth were subcultured in 96-hole culture plate 1×10^4/100μL per hole for 24 hours, the culture solution was changed into that of different concentrations of endostatin protein. There were three holes for every concentration of endostatin and negative controls. The cells were cultured in 37℃ 50mL/L CO_2 incubation for 48 hours. Then 50g/L MTT was added into the culture solution at 20μL/hole. Four hours later, the culture solution was drawn off, and 100μL DMSO was added into the solution. Then the culture plate was shaked on shaker for 5 minutes and optical absorption value was measured on immunodetection meter. The growth inhibition ratio was calculated by the following formula.

$$\text{Inhibition ration}=(1-\frac{A\text{ value of endostation group}}{A\text{ value of control group}})\times100\%$$

Statistical Analysis Curve fitting was simulated using SPSS 10.0 statistics software.

RESULTS

Identification, Proliferation and Purification of the Plasmids of pBlast-hEndostatin and pBlast-Mcs After pBlast-hEndostatin was digested with Nhe I and Sal I, two fragments of 5kb and 675bp were seen in 10g/L agar gel electrophoresis (Figure 1), suggesting that there was 675bp base pairs between polyclonal sites of Nhe I and Sal I. A fragment of 577bp was found in the PCR product using pBlast-hEndostatin as template, which corresponded to the sequence of pBlast-hEndostatin. No product was noted in the PCR reaction performed using pBlast-Mcs as template (Figure 2).

The PCR product was cloned into the carrier of PMD18-Vector and was sequenced. The result was as expected.

After the identified plasmid was amplified in Escherichia coli DH5α, it was extracted and purified with QIAGEN endofree plasmid maxi kit and analyzed with a ultraviolet spectrophotometer after being diluted 100 times. The $A260/A280$ ratio of pBlast-hEndostatin preparation was 1.9, and the pBlast-Mcs was 1.89.

Characteristics of Human Ocular Fibroblast Cultured *in vitro* After the fibroblasts were cultured about one day, the cells began to grow adherently with fusiform shape and propagated quickly with cells confluence in two weeks and arrangement in radiation and turbulence pattern. In HE staining, the cells were of fusiform shape and irregular triangle with abundant cytoplasm and oval nucleus in the cell center (Figure 3). It was positive for vimentin immunohistochemistry staining (Figure 4).

pBlast -hEndostatin and pBlast -Mcs Transfection, Screening and Identification pBlast-hEndostatin and pBlast-Mcs were transfected into human fibroblasts with Lipofectamine 2000. After the transfected cells were screened by Blasticidin (2mg/L)for 7 days, resistant cell clones appeared, which showed that pBlast-hEndostatin was successfully transfected and Blasticidin resisting gene was expressed. Then cell total RNA was extracted from the transfected fibroblasts, and RT-PCR was performed. A strap of 214bp was seen in 1.2g/L agarose gel electrophoresis (Figure 5), which showed that the transfected endostatin gene was expressed on transcriptional level. A strap of 20ku was seen in Western blot of pBlast-hEndostatin transfected cells, while no strap in pBlast-Mcs transfected cells (Figure 6). Immunohistochemistry showed negative for pBlast-Mcs transfected cells, and positive for pBlast-hEndostatin transfected cells.

Purification of Endostatin Protein The endostatin produced by above transfected human fibroblast was purified with ultrafiltration and affinity chromatography. The peak was the 2-3 sample tubes in 1mol/L NaCl eluate detected by ultraviolet spectrophotometer. There was an apparent protein strap in 20ku by SDS-PAGE. Western blot showed the 20ku strap which was human endostatin protein.

Proliferation-inhibiting Action of Endostatin on Human Umbilical Vein Endothelial Cells and Fibroblasts Under inverted microscope, the human umbilical vein endothelial cells were polygon and irregular shape. Twenty-four hours after the endostatin was added, the cells were gradually shrinking and became smaller. After MTT was added, many formazan crystals formed and covered the cells in control group, while few formazan crystals formed in endostatin group, which indicated that in endostatin group the vitality of the cells was poor.

Table 1 Inhibitory action of endostatin on human umbilical vein endothelial cells

Concentration of endostatin(mg/L)	Number of holes	*A* value	Inhibition ratio	IC50 value
Control group	3	0.480 ± 0.029		
2.5	3	0.439 ± 0.037	8.5%	
5	3	0.417 ± 0.025	13.1%	
10	3	0.347 ± 0.019	27.7%	34.5mg/L
20	3	0.297 ± 0.022	38.1%	
40	3	0.208 ± 0.022	56.7%	
80	3	0.174 ± 0.024	63.8%	

P=0.0002

Table 2 Inhibitory action of endostatin on human fibroblasts

Concentration of endostatin(mg/L)	Number of holes	*A* value	Inhibition ratio
Control group	3	0.206 ± 0.025	
2.5	3	0.206 ± 0.020	0%
5	3	0.212 ± 0.015	-3%
10	3	0.215 ± 0.013	-4%
20	3	0.206 ± 0.022	0%
40	3	0.213 ± 0.019	-3%
80	3	0.201 ± 0.014	2%

The inhibition ratios of endostatin of 2.5, 5, 10, 20, 40, 80mg/L on human umbilical vein endothelial cells at 48 hours were 8.5% ,13.1% ,27.7% ,38.1% ,56.7% ,63.8% respectively. By curve fitting, IC50 value of endostatin on human umbilical vein at 48 hours was 34.5mg/L (Table 1), while there was no inhibitive action of endostatin on human fibroblasts (Table 2).

DISCUSSION

In the current study, our results of restriction enzyme digestion, PCR reaction, DNA sequencing and the comparison of the sequence to genebank by NCBIBLAST software illustrated that pBlast-hEndostatin contained human endostatin gene of 555bp in size that was situated between Nhe I and Sal I sites.

The appearance of DNA recombination technique provokes human being the thought of gene therapy. Positive ion liposome can combine with DNA with negative charge to form electropositive compounds. These compounds are very easy to combine with electronegative cellular membrane and enter cells by endocytosis. So it has higher transfection efficiency.

The results of RT-PCR, immunohistochemistry and Western blot confirmed that transfected endostatin gene was transcribed and translated to produce full protein. All those data confirmed that pBlast-hEndostatin was successfully transfected into fibroblasts.

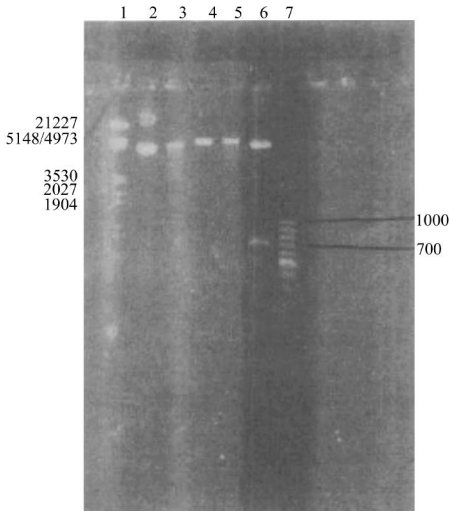

Figure 1 Restriction enzyme analysis of plasmids (Line1: Lam bda DNA/Hind III+EcoR I markers; Line2: pBlast-Mcs; Line3: pBlast-hEndostatin; Line4: pBlast-Mcs digested with Sal I; Line5: pBlast-hEndostatin digested with Sal I; Line6: pBlast-hEndostatin digested with Nhe I and Sal I; Line7: 100bp DNA ladder)

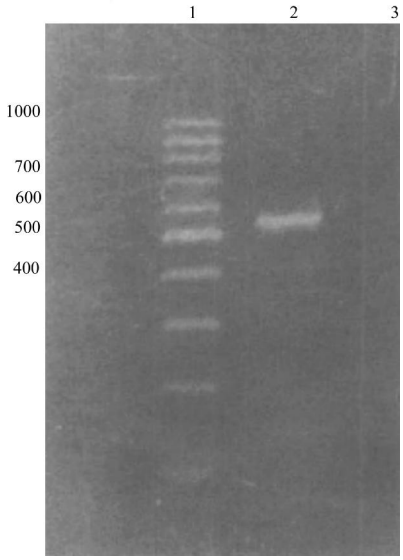

Figure 2 PCR results of plasmids (Line1: 100bp DNA ladder; Line2: result of pBlast-hEndostatin PCR; Line3: result of pBlast-Mcs PCR)

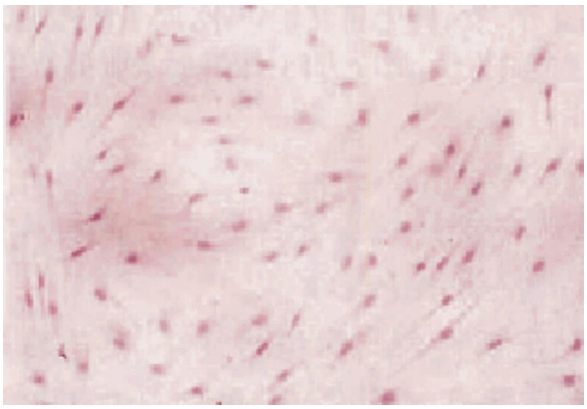

Figure 3 The cells were of fusiform shape and irregular triangle with abundant cytoplasm and oval nucleus in the cell center(HE×100)

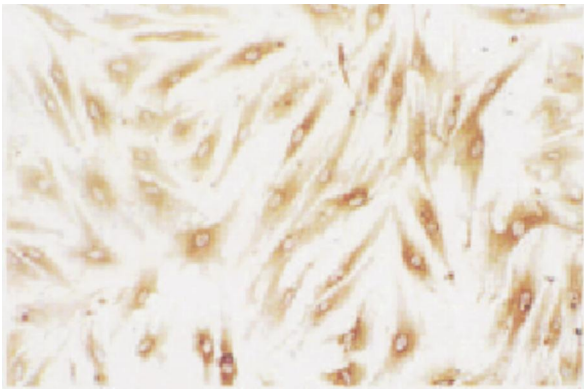

Figure 4 The cells were positive for vimentin immunohistochemistry staining ×200

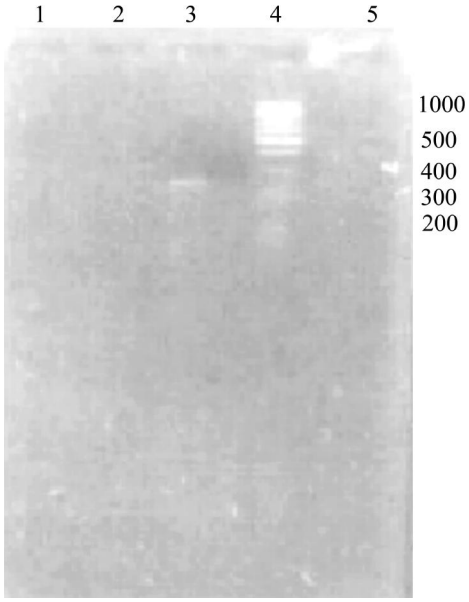

Figure 5 RT-PCR results of transfected fibroblasts (Line2: result of transfe cted pBlast-Mcs; Line 3:result of transfected pBlast-hEndostatin; Line4:100bp DNA ladder)

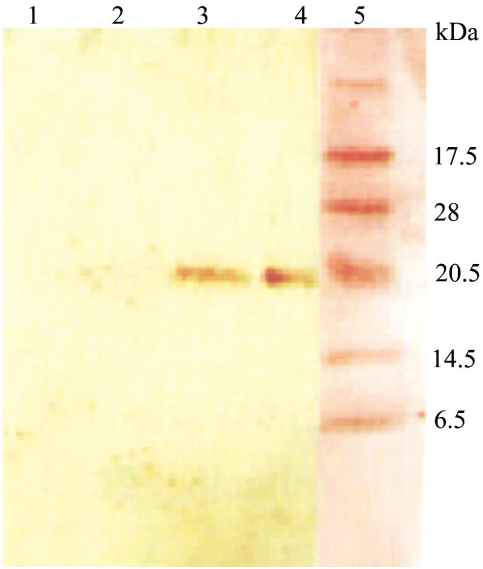

Figure 6 Western blot results of transfected fibroblasts (Line1,2: result of transfected pBlast-Mcs; Line3,4:result of transfected pBlast-hEndostatin; Line5:protein marker)

Molecular weight of endostatin is 20ku. Hohenester *et al*[1] analyzed the crystal structure of endostatin and found that it has high affinity with heparin. So we used ultrafiltration system of 50ku and 5ku to preliminarily separate cell lysate and culture supernatant. Then we used Hi-trap Heparin affinity column to further purify and gain preliminarily purified endostatin protein.

Endostatin was first discovered and purified from serum of murine suffered from hemangioendothelioma by O'Reilly *et al* [2] in 1997, and is recognized as one of the most potent antiangiogenic inhibitors. It was identified as a COOH-terminal proteolytic fragment of collagen of 184 amino acids, and has a molecular weight of 20ku. It specifically inhibits proliferation of endothelial cells, and induces endothelial cell apoptosis. It has no toxic side effects nor drug-resisting after repeated use[3-5].

The inhibitory results of endostatin on vascular endothelial cells were reported differently by different researchers. Blezinger *et al* [6] transfered human endostatin and mouse endostatin into human umbilical vein endothelial cells, the endostatin concentration at 48 hours in the culture supernatant was 5μg/L. The inhibition ratio of 1μg/L of endostatin on human lung-derived microvessel endothelial cells was 50%. Dhanabal *et al* [7] found that human endostatin protein had no inhibitive action on calf pulmonary artery endothelial cell, but had stronger inhibitive action on human umbilical vein endothelial cells and human microvascular endothelial cells than mouse endostatin protein. The inhibition ratio of 10mg/L human endostatin on human microvascular endothelial cell-lung was 64%, and IC50 was 5-7.5mg/L. Yoon *et al* [8] found that the inhibition ratio of concentrated (5 times) supernatant of mouse endostatin on human umbilical vein endothelial cells at 72 hours was 40%. He *et al*[9] found that IC50 of purified human endostatin protein on human umbilical vein endothelial cells at 72 hours was 72mg/L. We found that IC50 of purified human endostatin protein on human umbilical vein endothelial cells at 48 hours was 34.5mg/L. So the inhibitive action of endostatin on vascular endothelial cells in different laboratory was different. We think that the difference was because of different experiment methods. The difference may also come from recombinated endostatin proteins which were not processed and folded correctly [10]. In addition, in the process of purification the activity of endostatin often decreases obviously. Our experiment showed that endostatin protein had no inhibition on fibroblasts, which demonstrated that endostatin protein had specific inhibitive action on vascular endothelial cells. Our experiment also showed that endostatin gene can be transfected into human fibroblasts, which can produce endostatin protein. This result establishes the base of gene therapy for eye diseases related to neovascularization.

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