Comparative Study on *Trichoplusia ni* Tn 5B1-4 Cells and *Bombyx mori* BmN Cells for Recombinant Endostatin Production

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The recombinant plasmids harboring a heterologous gene coding mouse endostatin were transfected and expressed stably in *Trichoplusia ni* Tn 5B1-4 cells and *Bombyx mori* BmN cells, respectively. Recombinant endostatin expressed in the stably transformed Tn 5B1-4 and BmN cells was secreted into the medium. BmN cells are relatively lower in maximum cell growth and recombinant endostatin production than Tn 5B 1-4 cells. Recombinant endostatin was also purified to homogeneity using a simple one-step Ni^{2+} affinity fractionation method. Purified recombinant endostatin inhibited endothelial cell proliferation in a dose-dependent manner. The concentration at half-maximum inhibition (ED_{50}) for recombinant endostatin was approximately 0.35 μg/ml.

Key words: Mouse endostatin, *Trichoplusia ni* Tn 5B1-4 cells, *Bombyx mori* BmN cells, *In vitro* activity

Introduction

Angiogenesis is a process of blood vessel formation in which new vessels develop from existing vessels (Diaz-Flores *et al.*, 1994). Studies have shown that tumor growth is dependent on angiogenesis (Kerbel *et al.*, 1997). This provides a rationale for anti-angiogenic therapy in cancer. Recently, O'Reilly *et al.* (1997) have isolated endostatin, an angiogenesis inhibitor, from a murine hemangiothelioma cell line. The NH_2-terminal sequence of endostatin corresponds to the COOH-terminal portion of collagen XVIII. Endostatin is a specific inhibitor of endothelial cell proliferation and angiogenesis. Systemic administration of the purified recombinant endostatin has been shown to inhibit the growth of established tumors and metastases *in vivo* (O'Reilly *et al.*, 1997; Blezinger *et al.*, 1999). Endostatin does not induce acquired drug resistance after several treatment cycles (Boehm *et al.*, 1997).

An efficient expression system needs to be developed to produce the large quantities of soluble endostatin necessary for clinical trials. In this respect, endostatin is expressed in *E. coli* (O'Reilly *et al.*, 1997), *Pichia pastoris* (Boehm *et al.*, 1999; Duan *et al.*, 1999), human endothelial cells (Blezinger *et al.*, 1999) and *Drosophila melanogaster* S2 cells (Park *et al.*, 1999; Park *et al.*, 2001). Endostatin is also transiently expressed in *Spodoptera frugiperda* 21 (O'Reilly *et al.*, 1997) using a baculovirus expression system; however, continuous endostatin expression by stably transformed lepidopteran insect cells has not been examined yet. In this study, we compared the stable expression of cDNA coding for mouse endostatin in lepidopteran insect cell line Tn 5B1-4 or BmN and investigated *in vitro* activity of recombinant endostatin from stably transformed insect cells.

Materials and Methods

Cell line, plasmids and enzymes

Trichoplusia ni BTI Tn 5B1-4 (Tn 5B1-4) and *Bombyx mori* BmN cells were grown at 27°C in Nunc (T-25; Roskilde, Denmark) flasks in Sf900II-SFM (serumfree medium; Gibco BRL, USA) medium and TC-100 (contained 10% FBS) medium. The plasmid pIZT/V5-His (3.3 kb; Invitrogen, USA) contains an OpIE2 promoter, a V5 epitope tag, a polyhistidine region, and a zeocin resistance gene under the control of the EM7 promoter. The plasmid

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containing cDNA which encodes mouse endostatin was pMT/BiP/E/V5-His (Park *et al.*, 1999). *E. coli* DH5α was used as the primary host for constructing and propagating plasmids. *E. coli* cells were routinely grown and maintained in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.3) containing 50 μg zeocin/ml (Invitrogen) with agitation at 37°C. We used DNA restriction enzymes from Promega (USA) or Takara (Japan) according to the manufacturers instructions.

Construction of expression plasmids

The mouse endostatin sequence attached to *Drosophila* BiP signal sequence was amplified from plasmid DNA, pMT/BiP/E/V5-His by PCR using oligonucleotide primers. The sense primer was 5'-GGTACCATGGGAAT-GAAGTTATG-3' and the antisense primer was 5'-CCGCGGTTTGGAGAAAGAGG-3'. The amplified endostatin sequence was then inserted into the pGEM-T vector (Promega) to yield pGEM-T-BiP/E, and confirmed by DNA sequencing. PCR steps were performed in a Thermal Cycler (PE Biosystems, USA) using PCR mix (Takara) in a 50 μl volume. pIZT/BiP/E/V5-His (Fig. 1) was constructed by inserting a *Kpn*I-*Sac*II fragment of pGEM-T-BiP/E between the *Kpn*I and *Sac*II sites of pIZT/V5-His. The proper orientation and reading frame of the gene inserted in the recombinant plasmids of pIZT/BiP/E/V5-His were confirmed by both restriction enzyme mapping and DNA sequencing.

Stable transformation

Exponentially growing Tn 5B1-4 and BmN cells were transfected with plasmid pIZT/BiP/E/V5-His using the lipofectin method. To prepare the transfection medium, plasmid DNA and lipofectin reagent (Gibco BRL) were separately diluted with Sf900II-SFM and TC-100, and then mixed together in a ratio of 1:5. The transfection medium was incubated at room temperature for 15 min
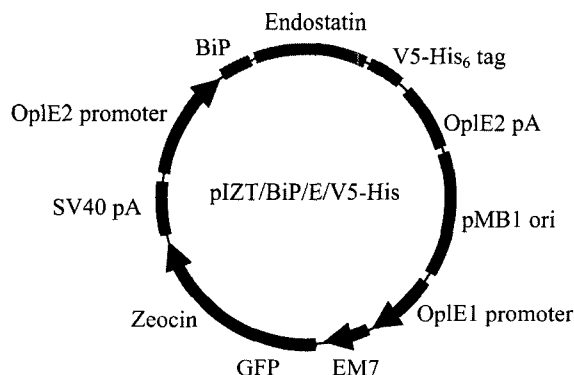

Fig. 1. Schematic representation of expression plasmid, pIZT/BiP/E-V5-His.

and transferred into 6-well plates pre-seeded 2 hrs earlier with Tn 5B1-4 cells and BmN. After 24 hrs of incubation, the medium was changed to remove the lipofectin and the cells were incubated for 4 more days in Sf900II-SFM and TC-100 without drug selection. Then, the medium was changed to selective Sf900II-SFM and TC-100 containing 400 μg zeocin/ml. The selective medium was replaced every 4 days. Stably transformed polyclonal cell populations were isolated after 4 weeks of selection with zeocin. Zeocin was maintained routinely with 200 μg/ml in the media at all times after selection.

Cell culture and analysis of gene expression

Stably transformed Tn 5B1-4 and BmN cells expressing endostatin were grown at 27°C in Nunc (T-25) flasks in 5 ml of Sf900II-SFM and TC-100 containing 200 μg/ml zeocin. Stably transformed Tn 5B1-4 and BmN cells were cultured for 8 days and 9 days in multiple Nunc (T-25) flasks to analyze cell growth and endostatin expression. Cultures were centrifuged at 2,000 × g for 5 min to separate the cells. The supernatant was used to identify extracellular recombinant proteins. The cell fraction was rocked for 1 hr in lysis buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg phenylmethylsulfonyl fluoride/ml, 1 μg aprotinin/ml, and 1% Triton X-100] and subjected to three freeze-thaw cycles between a −70°C freezer for 10 min and a 37°C water bath for 2 min. After centrifuging the cell extracts at 9,300 × g for 15 min to remove cell debris, the supernatant was used to identify intracellular recombinant proteins.

Purification of recombinant endostatin

All steps were carried out at 4°C. Polyhistidine-tagged endostatin was purified using His-Bind Kits (Novagen, Madison, WI, USA) in a chromatography column (Novagen), based on the manufacturers instructions. Briefly, the His-Bind resin slurry was washed with distilled water and Ni²⁺ cations were immobilized onto the resin using the charge buffer. Then the resin was equilibrated with binding buffer containing 5 mM imidazole. The medium fraction of the culture was incubated with the equilibrated resin for 1 hr at 4°C. Weakly bound proteins were washed from the resin by the binding buffer, and contaminating proteins still bound to the resin were removed by increasing the imidazole concentration to 100 mM. The recombinant polyhistidine-tagged protein product was finally eluted with the buffer containing 1 M imidazole. Protein concentrations were determined by the Bradford protein assay kit (Bio-Rad, USA) with bovine serum albumin as the standard.

Bovine capillary endothelial cell proliferation assay

The endothelial cell proliferation assay procedure was a

modification of the method of O'Reilly *et al.* (1997). Bovine capillary endothelial (BCE) cells (Clonetics, USA) were maintained in EGM containing 2% fetal bovine serum, 12 µg bovine brain extract/ml, 10 ng human epidermal growth factor/ml, 1 µg hydrocortisone/ml, 50 µg gentamycin/ml, and 50 µg amphotericin/ml, as suggested by the manufacturer (Clonetics). Approximately 2×10^4 cells in 0.5 ml of the EGM were added to each well of gelatinized 24-well plates and incubated at 37°C (in 5% CO_2) for 24 hrs. The medium was replaced with 0.25 ml of fresh EGM (described above) with antibiotics, and different concentrations of recombinant endostatin were added to each well. After 30 min of incubation, the cells were stimulated with 1 ng bFGF/ml, and the EGM with antibiotics was added to obtain a final volume of 0.5 ml. After 72 hrs of incubation, the cells were trypsinized and counted using a hemacytometer. To ensure that any inhibition observed was not due to detachment of the BCE cells from the plate, all wells were examined for evidence of the cell detachment under an inverted microscope prior to cell counting. The endothelial cell proliferation assay was replicated three times. The concentration at half-maximum inhibition (ED_{50}) was estimated by probit analysis (Finney, 1971).

Western blot analysis

Protein samples were analyzed by SDS-PAGE according to the Laemmlis method (Laemmli, 1970). The electrophoresed proteins on the gel were transferred onto nitrocellulose, incubated with mouse anti-V5 (Invitrogen) polyclonal antibody, and probed with rabbit anti-mouse IgG alkaline phosphatase conjugate (1:1000 v/v). After washing, BM purple AP substrate solution (Boehringer Mannheim, Germany) was added and the reaction was quenched with distilled water.

Results and Discussion

Analysis of gene expression

We examined the expression of endostatin in stably transformed Tn 5B1-4 and BmN cells. Gene expression was analyzed after 5 days of cultivation by Western blot analysis. Recombinant endostatin protein was detected in transformed Tn 5B1-5 and BmN cells carrying pIZT/BiP/E-V5-His by Western blot analysis with mouse anti-V5 polyclonal antibodies (Invitrogen), and had a molecular weight of ~25 kDa. This molecular size approximated to the predicted molecular weight of fused endostatin containing the C-terminal tag of V5 and His_6 (data not shown).
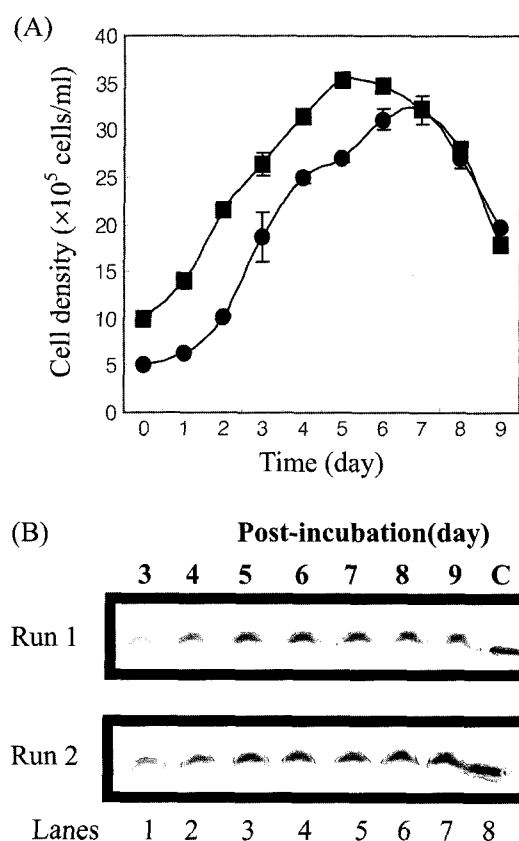

Fig. 2. Time-course changes in cell growth and recombinant endostatin expression during T-flask cultures of transformed BmN cells. (A) The cell concentrations are plotted against the incubation time for runs 1 (squares) and 2 (circles). (B) For runs 1 and 2, recombinant endostatin expression at incubation times of 3, 4, 5, 6, 7, 8 and 9 days was confirmed by Western blot analysis and is shown in lanes 1, 2, 3, 4, 5, 6 and 7, respectively. Lane 8, labeled "C" contains 0.2 µg of purified endostatin control.

Time-course changes in cell growth and recombinant endostatin expression

The time-course changes in cell density and recombinant endostatin expression were examined during T- flask cultures of transformed Tn 5B1-4 and BmN cells. The maximum cell density of transformed Tn 5B1-4 cells was 5.7 $\times 10^6$ cells/ml at 6 days of cultivation for the run with a seeding density of 5×10^5 cells/ml (data not shown). On the other hand, the maximum cell density of transformed BmN cells was 3.54×10^6 cells/ml (Fig. 2A). In Western blot analysis, recombinant endostallstin production of transformed Tn 5B1-4 cells for the run with a seeding density of 1×10^5 cells/ml was estimated to be approximately 4.6, 9.3, 12.6, 13.6, and 14.1 mg/L 4, 5, 6, 7, and 8 days after seeding, respectively, in comparison to a purified endostatin control. Similarly, recombinant endostatin production for the run with a seeding density of 5×10^5

Table 1. Comparison of Tn 5B 1-4cells and BmN cells

	Tn 5B 1-4	BmN
Maximum cell growth ($\times 10^5$ cells/ml)	57.3	35.4
Endostatin expression level (mg/L)	14.3	3.6
Specific content (μg /10^6 cells)	2.50	1.02

cells/ml was estimated to be approximately 12.6, 14.0, 14.3, 14.1, and 13.7 mg/L 4, 5, 6, 7, and 8 days after seeding, respectively. The maximum recombinant endostatin production was 14.3 mg/L at a seeding density of 5×10^5 cells/ml at 6 days after cultivation (data not shown). Transformed BmN cells at initial cell concentration of 1×10^6 cells/ml for 6 days produced about 3.6 mg recombinant endostatin/L (Fig. 2B), this is quarter of the content produced by transformed Tn 5B1-4 cells, and in specific content transformed Tn 5B1-4 cells produced 2.5 times higher (Table 1). Thus, these results show that transformed Tn 5B1-4 cell system is better than transformed BmN cell system in the production of useful proteins.

In the case of transformed Tn 5B1-4 cells, the level (14.3 mg/L) of recombinant protein expression is higher than that (10.2 mg/L) for the expression of the juvenile hormone esterase from the transformed Hi5 cells (Farrell et al., 1998) and lower than that (22.8 mg/L) for the expression of the granulocyte-macrophage colony stimulating factor from the transformed Hi5 cells (Keith et al., 1999). However, it is much higher than that (1 – 2 mg/L) reported for the expression of mouse endostatin from baculovirus-infected *Spodoptera frugiperda* 21 cells (O'Reilly et al., 1997). This shows the potential of the transformed insect cell-expression system for recombinant protein production, as compared to the baculovirus infected-insect cell system.

Purification of the his-tagged endostatin

Using transformed Tn 5B1-4 cells which showed better in recombinant endostatin expression than transformed BmN cells, purification was conducted to measure *in vitro* activity of recombinant endostatin. Recombinant endostatin was purified rapidly to near homogeneity by a simple one-step Ni^{2+}-chelation affinity purification procedure as described in Materials and Methods. Protein purity was analyzed by SDS-PAGE and silver staining (Fig. 3A). Western blot analysis further confirmed the identity of the purified protein (Fig. 3B). A total of 300 µg of purified polyhistidine-tagged endostatin (Endostatin-V5-His_6) was obtained from the medium fraction of a 220 ml culture without visible contaminating proteins on a silver nitrate-stained SDS-PAGE gel.

Bovine capillary endothelial cell proliferation assay

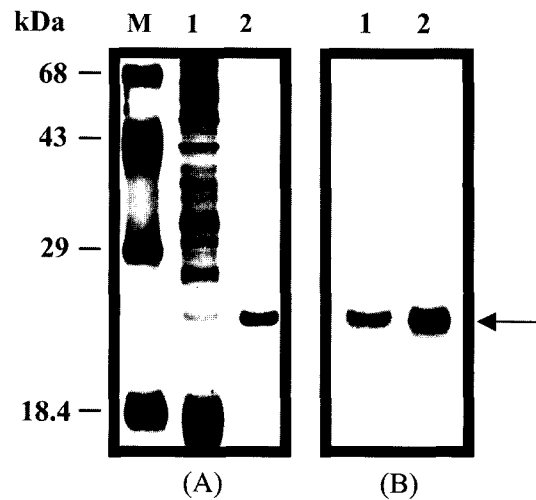Endostatin, an angiogenesis inhibitor, suppresses endot-

Fig. 3. SDS-PAGE (A) and Western blot analysis (B) of the purification of the his-tagged endostatin from the medium fraction of stably transformed Tn 5B1-4 cell culture. M indicates the molecular weight markers. Before and after affinity purification of the medium fraction of stably transformed Tn 5B1-4 cells is shown in lanes 1 and 2, respectively. The arrow indicates the recombinant endostatin protein.

Table 2. Inhibition of endothelial cell proliferation by recombinant endostatin

Endostatin content (µg/ml)	Cell density ($\times 10^4$ cells/ml)*
0	10.13
0.1	7.56
0.2	5.62
0.4	4.69
0.6	3.81
0.8	3.44
1.0	3.38
1.2	3.19
1.4	2.94
1.8	2.94
2.4	2.69

*The average of triplicate runs was reported.

helial cell growth *in vitro* (O'Reilly et al., 1997). The effect of purified recombinant endostatin on suppression of bFGF-stimulated endothelial cell proliferation was examined to determine whether this protein is biologically active. Recombinant endostatin inhibited the proliferation of bovine capillary endothelial cells in a dose-dependent fashion (Table 2). The presence of a his-tag and the VP5 epitope sequence did not affect suppression of recombinant endostatin on bFGF-stimulated endothelial cell proliferation. The concentration at half-maximum inhibition (ED_{50}) for recombinant endostatin was approximately 0.35 µg/ml. This ED_{50} is higher than the level (0.28 µg/ml) reported for native mouse endostatin (O'Reilly et al.,

1997). Our result shows that a biologically active form of recombinant endostatin expressed from Tn 5B1-4 cells has anti-proliferative action on bovine endothelial cells.

When expressed in stably transformed Tn 5B1-4 and BmN cells under the influence of the *Drosophila* BiP protein signal sequence, recombinant endostatin was found primarily in the medium fraction. Optimal production of recombinant endostatin was obtained from stably transformed Tn 5B1-4 and BmN cells at 6 days after cultivation. Transformed Tn 5B1-4 showed four times higher in recombinant endostatin production, compared with transformed BmN cells.

The purified, biologically active form of recombinant endostatin expressed from Tn 5B1-4 cells has anti-proliferative action on bFGF stimulated-bovine endothelial cells.

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