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Anti-Angiogenic Activity of Mouse N-/C-terminal deleted Endostatin

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Endostatin, a proteolytic fragment of collagen XVIII, is a potent inhibitor of angiogenesis and the growth of several primary tumors. However, the opinions on the activity of endostatin derivatives deleted N- or C- terminal are still controversial. In this regard, we produced mouse endostatin and its derivatives in the prokaryotic system, and studied their anti-tumor activity. The [^{3}H]-thymidine incorporation assay demonstrated that N-terminal deleted mouse endostatin, and a C- and N-terminal deleted mutant, effectively inhibited the proliferation of human umbilical vein endothelial cells (HUVECs). The biological activity of endostatin was also shown by its *in vivo* anti-angiogenic ability on the chorioallantoic membrane (CAM) of a chick embryo. Treatment of 200 ng of mouse endostatin, or N-terminal deleted mouse endostatin, inhibited capillary formation of CAM 45 to 71%, which is comparative to a 80% effect of positive control, 1 μg of retinoic acid. An *in vivo* mouse tumor growth assay showed that N-terminal deleted mouse endostatin, and the N-/C-terminal deleted mutant, significantly repressed the growth of B16F10 melanoma cells in mice as did the full-length mouse endostatin. According to these results, N- and N-/C-terminal deleted mouse endostatins are the potent inhibitors of tumor growth and angiogenesis.

Keywords: Endostatin, Angiogenesis

Introduction

Angiogenesis, the generation of new blood vessels from pre-existing capillaries, is important in normal physiological processes such as embryonic development, wound healing, and organ and tissue regeneration. It also plays a pivotal role in tumor progression and metastasis. Tumor cells produce several angiogenic factors, including the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF) (Senger *et al.*, 1983, Folkman *et al.*, 1995, Jo *et al.*, 1999, Lee *et al.*, 1998). On the other hand, many malignant tumors also generate inhibitors of angiogenesis, such as thrombospondin-1 and angiostatin (Dameron *et al.*, 1994; Ambs *et al.*, 1998; Gateley *et al.*, 1996). Recently a murine 20 kDa fragment of collagen XVIII, endostatin, was identified (O'Reilly *et al.*, 1997). Endostatin suppresses the proliferation of endothelial cells and the growth of several primary tumors. Nonetheless, the action mechanism of endostatin has not yet been defined. However, given the high affinity of endostatin for heparin, interference with the heparan sulphate requirement of bFGF signaling is a plausible possibility (Hohenester *et al.*, 1998).

Another possibility is to bind the receptor of VEGF, and prevent its activation of downstream cascade. Yamaguchi *et al* (1999) hypothesized that endostatin blocks in one or more steps in VEGF-mediated cell migration.

Recently the structure of endostatin has been published. However, the results concerning the role of the zinc-binding site (histidine-1, -3, and 11 and a fourth ligand, aspartic acid-76) are unclear and controversial (Ding *et al.*, 1998; Boehm *et al.*, 1998; Yamaguchi *et al.*, 1999; Hohenester *et al.*, 2000). Yamaguchi *et al.* (1999) suggested that the inhibition activity of endostatin against migration would be unaffected by the mutations eliminating zinc or the heparin binding site; and that the inhibition of tumor growth does not depend on zinc-binding. On the other hand, Boehm *et al.* (1998) reported that the H1/3A double mutant, H11A, and D76A single mutants of endostatin were unable to regress the growth of Lewis lung carcinoma.

Here we examined the activity of the deletion mutants of mouse endostatin in order to gain insight for developing more selective and powerful anti-angiogenic inhibitors.

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Materials and Methods

Expression and purification of endostatin and its derivatives Mouse endostatin and its derivatives were produced in a prokaryotic system, *E. coli*. First, the endostatin mRNA was amplified by RT-PCR. Also, endostatin cDNA also amplified by PCR using the following primers: 5'-CACAGCCACCGCGACTTC CAG-3', 5'-CTACTTGGAGGCAGTCATGAA-3'. PCR was carried out for 30 cycles with the following parameters: 94°C denaturation for 1 min, 54°C annealing for 1 min, and 72°C elongation for 1 min.

In the next step, we treated different kinds of restriction enzyme and T-vector (Promega). These restricted endostatin sequences were auto-sequenced (ABI prism 377 model). On the basis of the sequencing result, we produced primers for endostatin derivatives. Primers for F5 are as follows: 5'-TTCCAGTCTTCAGCAGGCG-3', 5'-CGTCCGCCACGRCT CACAGTA-3'. Primers for M3 are the following: 5'-CAGCAGGCGCGGGCCGRG-3', 5'-CTACTTG GAGGCAGTCATGAA-3'. PCR was carried out for 30 cycles with the following parameters: 94°C denaturation for 1 min, 54°C annealing for 1min, and 72°C elongation for 1 min.

Mouse endostatin and the N-terminal deletion mutant, M3, were His-tagged at their N-terminal. Following Glutathion-*S*-Transferase (GST), the tagged endostatin derivatives were cloned into *Eco*RI/*Sal*I sites of the pGEX-5x-1 vector (Amersham Pharmacia Biotech) and produced the following: (1) full-length mouse endostatin, GF; (2) C- and N-terminal deleted mutant, F5. Also, several His-tagged mouse endostatin derivatives were designed through deleting the N- and/or C-terminal and cloned into *XhoI/BamHI* sites of the pET-15b vector (Novagen). These proteins are described in Fig. 1.

Each endostatin derivative-containing vector was transformed into *E. coli* and a random selection was carried out. The colony having good expression was chosen and grown at a large scale of 250 ml. Most endostatin and endostatin derivative proteins were expressed at the yield of 10-20 mg/ml. Both GST- tagged and His-tagged derivatives were purified as insoluble forms. Because of the insoluble characterization of endostatin, we first carried out centrifugation. After the centrifugation, the purity of the GST-tagging protein was checked by SDS-PAGE and it was almost 80%. His-tagged proteins were purified in the denatured condition using a Ni-NTA column (QIAGEN) in the secondary purification step. The purity of these proteins was up to 99%. Proteins produced as an insoluble form were treated with a 8 M solution of urea. Then, urea was removed through repeated dialysis against the PBS buffer to obtain the refolding of the proteins.

Cell culture Human umbilical vein endothelial cells (HUVECs) were obtained from a human umbilical cord vein by the method of Jaffe (Jaffe *et al.*, 1973). The cells were grown onto a gelatin-coated 75 cm^2 flask in M199 with a 20% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml bFGF, and 5 units/ml heparin. The HUVECs used in this study were from passages 2 to 7. Hepatoblastoma HepG2 cells were maintained in a Modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. All of the cells were cultivated at 37°C under 5% CO_2, 95% air.

[^{3}H]-Thymidine incorporation assay The HUVECs or HepG2 cells were seeded at a density of 2×10^4 cells/well in a 24-well plate. Cells were incubated in a corresponding growth media and allowed to attach for 24 h. Cells were washed twice with M199 and incubated for 6 h with M199 containing 1% FBS. Cells were stimulated by the addition of 5 ng/ml of bFGF with or without H5 or endostatin for 30 h, followed by the addition of 1 μCi/ml [^{3}H]-thymidine for 6 h. High molecular mass [^{3}H]-radioactivity was precipitated using 5% trichloroacetic acid at 4°C for 30 min. After two washes with ice-cold H_2O, [^{3}H]-radioactivity was solubilized in a 0.2 N solution of NaOH containing 0.1% SDS, and determined by a liquid scintillation counter (Beckman Instrument). All of the experiments were fulfilled in triplicate and repeated more than twice.

Chorioallantoic membrane assay (CAM assay) The angiogenic assay with a chick chorioallantoic membrane (CAM) was performed as described (Brooks *et al.*, 1994; Friedlander *et al.*,

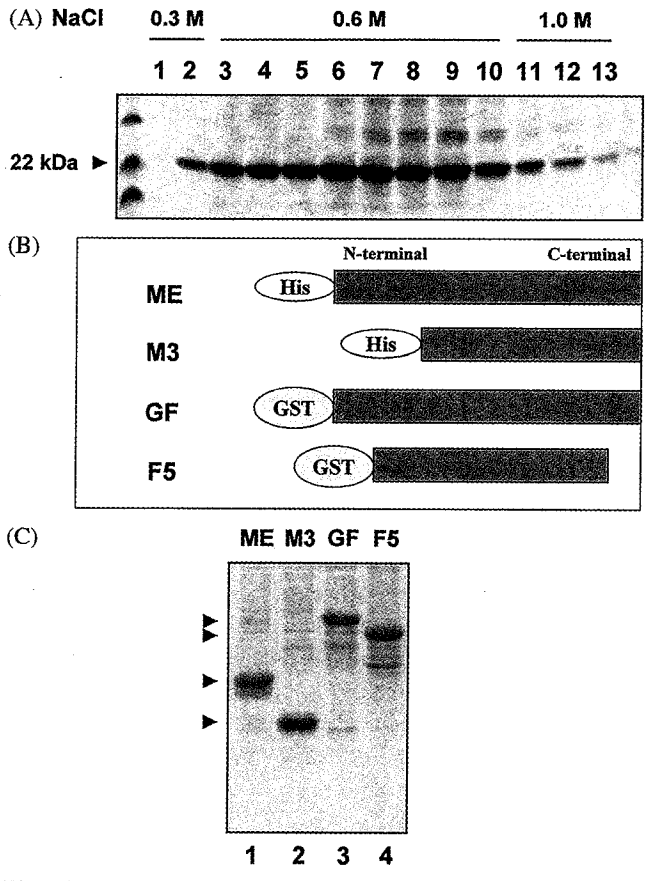

Fig. 1. Diagram of endostatin and its derivatives. Mouse endostatin (22 kDa) was purified in a 0.6 M solution of NaCl using a heparin column (A). From mouse endostatin, four derivatives were manufactured: (1) full length mouse endostatin bound histidine at the N-terminal (ME). (2) The N-terminal deleted mutant (M3: 35-184 amino acid). (3) A full-length mouse endostatin bound GST at the N-terminal (GF). (4) The C- and N-terminal deleted endostatin derivative with GST at the N-terminal (F5: 31-140 amino acid) (B). Produced mouse endostatin and its derivative proteins were analyzed using SDS-Page analysis (C). The arrowhead indicates each of the isolated endostatin derivatives in the panel (C).

1995, Choi *et al.*, 1998). Fertilized chick embryos were preincubated for 5 days at 37°C with 70% humidity. A hole was drilled over the air sac at the end of the egg, and a vascular zone was identified on the CAM. A 1 × 1-cm window in the shell was made to expose the CAM. Thermanox coverslips were sterilized and loaded with endostatin, endostatin derivatives and retinoic acid were used as a positive control. After air-drying under the laminar flow, the coverslips were applied to the CAM surface of a 5-day-old chick embryo. The windows were sealed with clear tape, then the eggs were incubated for 2 more days. Intrapos (Green-Cross, Korea) was injected under the upper CAM to increase contrast between the vessels and background. Each capillary formation was inspected using a light microscope.

***In vivo* Anti-tumor growth assay** To investigate the effect of mouse endostatin and F5 on tumor growth, 1×10^6 B16F10 melanoma cells were injected into the sole of the C57BL/6J mouse foot (a 6-8 weeks old mouse). In this step, cultured B16F10 melanoma cells were treated with 0.9% PBS. The tumor within the mouse was grown to 5-8 mm in diameter after injection of B16F10 cells. Then 10 mg/kg/day of F5 and mouse endostatin were injected for 10 days. The tumor size was measured using slide caliper and observed with bare eyes as well as the microscope. In the negative control group we treated only PBS. We performed *In vivo* Anti-tumor Growth Assay using 4 mice/groups.

Results

Diagram of endostatins and their derivatives Mouse endostatin is a 22 kDa protein. It was isolated in a 0.6 to 1 M solution of NaCl by using a heparin column (Fig. 1A) and His-tagged mouse endostatin derivatives that were isolated by a Ni-NTA column.

Four endostatin derivatives were produced as described in Fig. 1B and Fig. 1C: (1) Full-length mouse endostatin bound histidine at the N-terminal (ME). (2) The N-terminal deleted endostatin mutant with histidine (M3: 35-184 amino acid). (3) A full-length mouse endostatin bound GST at the N-terminal (GF). (4) The C- and N-terminal deleted endostatin derivative with GST at the N-terminal (F5: 31-140 amino acid).

GST-tagged endostatin derivatives were cloned into a pGEX-5x-1 vector. Also, His-tagged mouse endostatin derivatives were designed through deleting the N- and/or C-terminal and were cloned into a pET-15b vector.

Mouse endostatin and its derivative F5 potently inhibited proliferation of HUVECs To confirm the selective activity of endostatin on endothelial cells, a proliferation assay was performed with HUVECs and HepG2 cells. To measure the proliferation quantitatively, [^{3}H]thymidine was treated into HUVECs. At first, proliferation of HUVECs was examined on either 5 ng/ml bFGF-treated or untreated media. As expected, endostatin and M3 inhibited the proliferation of endothelial cells dose-dependently (Fig. 2A). The addition of F5 at 10 μg/ml effectively inhibited the proliferation of the bFGF-treated HUVEC cells (Fig. 2B). In contrast,

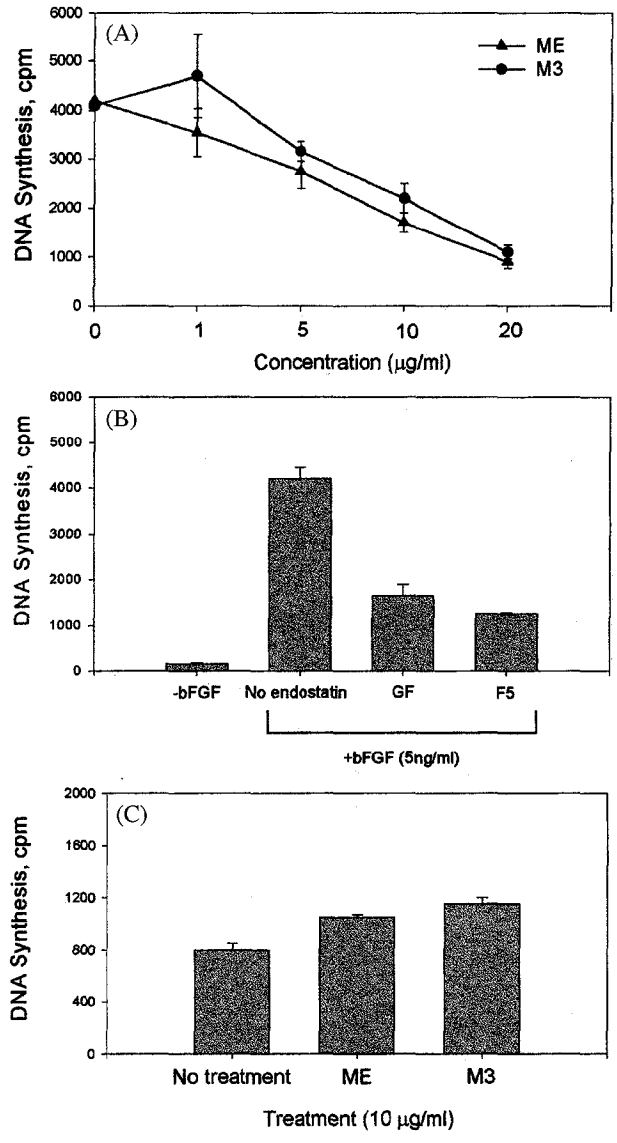

Fig. 2. Inhibition of endothelial cells by mouse endostatin and its derivatives. Each 10 ug/ml His-tagged mouse endostatin (ME: ▲), and His-tagged / N-terminal deleted endostatin M3 (●) were treated into the 5 ng/ml bFGF-treated HUVECs to analyze the proliferation of endothelial cells(A). The GST-tagged full-length mouse endostatin and F5 were also treated into the 5 ng/ml bFGF-treated HUVECs (B). HUVEC cells were significantly increased the proliferation by treating bFGF compared to the sample that was not treated with bFGF (B). Also, 10 ug/ml of ME and M3 were treated into the HepG2 cells (negative control) (C).

proliferation of HepG2 (hepatocellular carcinoma cells) was not suppressed by endostatin or M3 (Fig. 2C). This indicates that insoluble endostatin and its derivatives are selectively active on endothelial cells.

Angiogenesis on the chorioallantoic membrane (CAM) was inhibited by mouse endostatin and M3 To confirm the anti-angiogenic activity of mouse endostatin and its

Table 1. Angiogenesis in the CAM was potently inhibited by endostayin and M3

	Dose (ng/egg)	Inhibitor activity (%) (inhibited/total)	
Retinoic acid	1000	80	(28/35)
Negative control	0	18	(6/34)
Mouse endostatin	200	71	(10/14)
M3	200	45	(5/11)

Mouse endostatin and M3 were treated in 200 ng onto the CAM. Retinoic acid (RA) as a positive control was treated in the CAM with 1 μg volume. Negative control means that nothing was treated into CAM.

derivative M3, we carried out an *in vivo* CAM assay. As a positive control, retinoic acid (RA) was treated into the CAM. 1 μg of RA inhibited the CAM angiogenesis by 80%. Also, the treatment of 200 ng mouse endostatin, and their derivative M3, inhibited the angiogenesis by 71% and 45%, respectively (Table 1). Although endostatin and M3 inhibited angiogenesis in the CAM by 45-71%, the endostatin derivatives was treated by only 20% of the dose of RA (positive control), a potent angiogenesis inhibitor. According to this data, we suggest that the N-terminal deleted endostatin mutant also has anti-angiogenic activity.

Tumor growth was suppressed by endostatin derivatives in the *in vivo* assay To investigate the effect of mouse endostatin and its derivatives (F5 and M3) on *in vivo* tumor growth, B16F10 melanoma cells were injected into the sole of the C57BL/6J mouse foot. When the tumor was grown to 8 mm in diameter, we injected 10 mg/kg/day of mouse endostatin, F5 and M3, for 10 days. Compared to the control, tumor growth was significantly inhibited by M3, F5 and mouse endostatin (GF) (Fig. 3). Even though the suppression was incomplete, the inhibitory activities of mouse endostatin and M3 were comparably good during the experimental period of 20-days.

Discussion

In the original study by O'Reilly *et al.* (1997) recombinant mouse endostatin was produced in the baculovirus and *E. coli* expression systems. It demonstrated selective inhibition of endothelial cell proliferation *in vitro* and anti-angiogenic activity in the cell adhesion molecule assay. It also potently suppressed the growth of metastasis in a Lewis lung carcinoma model, but the underlying mechanisms by which endostatin inhibits endothelial cell proliferation and angiogenesis have not yet been determined. Human endostatin was cloned in 1999 (Dhanabal *et al.*, 1999), and the overall identity between mouse and human endostatin is 87%. Although a structural study with mouse endostatin was carried out intensively, the zinc-binding for endostatin activity and

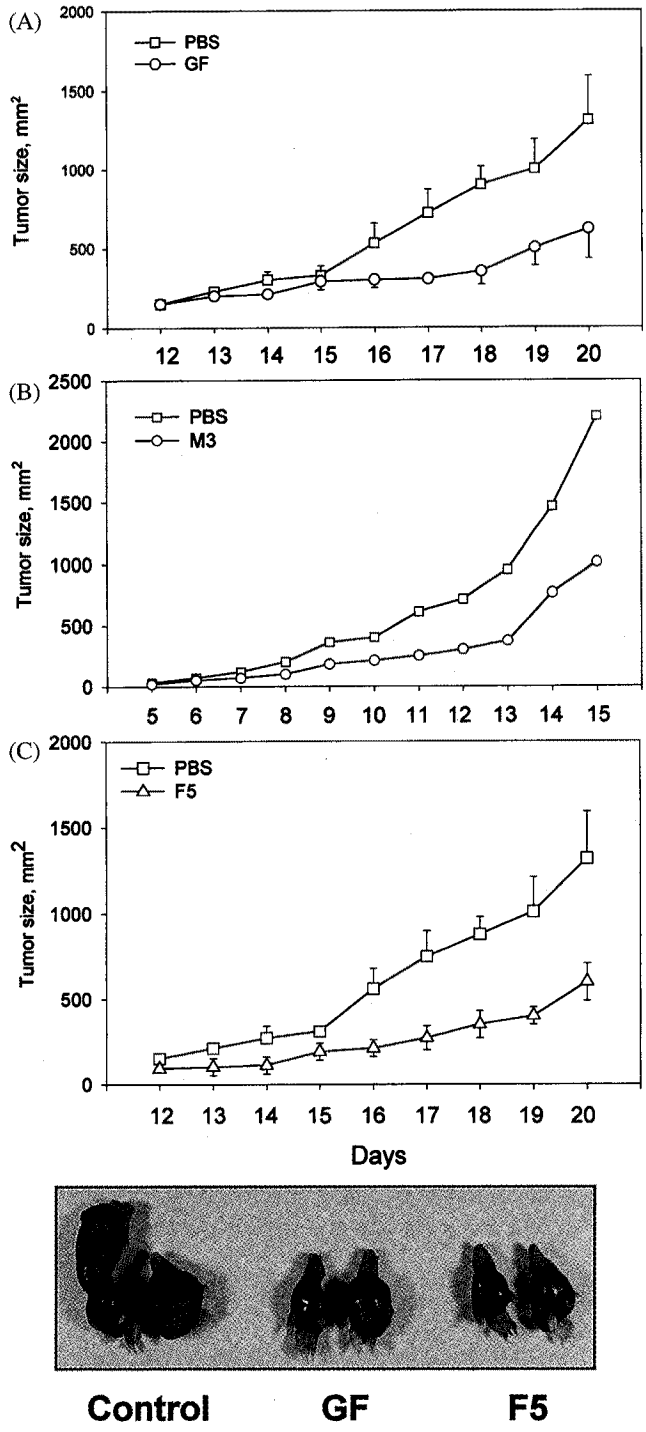

Fig. 3. Inhibition of the *in vivo* tumor growth by endostatin and mutant endostatins. To analyze the effect of endostatin and its derivatives against *in vivo* anti-tumor growth, 1×10^6 B16F10 melanoma cells were injected into the C57BL/6J mouse foot (6-8 weeks old mouse). And 10 mg/kg/day GF or M3 was injected for 10 days; F5 also was treated by the same method (4 mice/group). The tumor volume was measured using a slide caliper.

dimerization is still unclear. There were several reports on the importance of the N-terminal zinc-binding domain for the

anti-angiogenic activity. For instance, Standker *et al.* (1997) reported that the 12-aa shorter human endostatin was purified from plasma, but was ineffective in the *in vitro* angiogenesis assays. Also, Boehm *et al.* (1998) suggested that zinc-binding of endostatin is essential for its antiangiogenic activity. Another result using C- and N-terminal deleted mutants showed that regardless of the zinc-binding, endostatin inhibits VEGF-induced endothelial cell migration and tumor growth (Yamaguchi *et al.*, 1999). Current studies on endostatin concentrate on seeking the action mechanism of endostatin and confirming the activity of endostatin for clinical therapy (Dhanabal *et al.*, 1999; Dixelius *et al.*, 2000; Kim *et al.*, 2000; Sasaki *et al.*, 2000).

In our study, mouse endostatin strongly inhibited the proliferation of endothelial cells, but not that of non-endothelial cells, HepG2. These results are consistent with other reports that the proliferation on non-endothelial cells was not inhibited by endostatin (Dhanabal *et al.*, 1999). Furthermore, endostatin derivative F5 suppressed the bFGF-induced proliferation of HUVECs at the concentration of 10 μg/ml. We also showed that mouse endostatin and M3 inhibited the capillary formation in CAM assay by 45-71%. The C- and N-terminal deleted mutants, F5, also had strong inhibition activity against *in vitro* and *in vivo* angiogenesis and tumor growth. F5 may exist as a monomeric form since it does not have a zinc-binding domain, which means that an active endostatin does not need to be a dimer (Ding *et al.*, 1998). These data with F5 can be interpreted that the zinc-binding site is not essential for the anti-angiogenic activity of endostatin. Even if we cannot specify the role of the C- and N-terminal of endostatin, these regions do not appear effective in augmenting the anti-angiogenic activity of endostatin. However, the structure activity relationship of each domain of endostain has yet to be studied.

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