Effects of the C-terminal of endostatin on the tumorigenic potential of H22 cells

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Abstract. Endostatin is an endogenous angiogenesis inhibitor whose specific functional site has not yet been determined. In the present experiment, 13 amino acids (LCIENSFMTSFSK) were selectively deleted from the C-terminal of endostatin and the resulting mutant endostatin was named EM13. To determine the effect of the C-terminal deletion on the biological activity of endostatin, EM13, wild-type endostatin and empty plasmid were transfected into H22 cells. After 48 h, the three types of transfected cells were harvested and injected into nude mice. The results demonstrated that there was no significant difference in tumor size, as determined by hematoxylin and eosin staining, between the EM13-transfected group and the endostatin and empty plasmid groups, although the nude mice that were injected with EM13-transfected H22 cells exhibited smaller tumors and lower density of blood vessels compared to those injected with endostatin- and empty plasmid-transfected H22 cells. The results suggested that the 13 amino acids of the C-terminal of endostatin do not play an important role in the tumorigenic potential of H22 cells. This experiment was unsuccessful in reproducing the results of several investigators. Therefore, the mechanism underlying the tumorigenesis of H22 cells remains to be elucidated.

Introduction

Angiogenesis is the formation of new capillaries from preexisting blood vessels and it is an important mechanism involved in various pathological processes, including inflammation and tumor growth. Antiangiogenic therapy is being investigated as a potentially powerful novel therapy for cancer and other angiogenesis-dependent diseases.

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Endostatin is one of the most potent inhibitors of angiogenesis and may induce tumor regression in mice (1,2). Clinical trials on the antitumor effects of endostatin are currently ongoing (3). Originally, endostatin was purified from a conditioned medium of murine hemangioendothelioma cells as a proteolytically cleaved fragment of type XVIII collagen. The generation of endostatin may be achieved by cleavage of collagen by cathepsin L (4), matrilysin (5) or elastase (6). Endostatin activated by proteolytic processing (7) may inhibit endothelial cell proliferation, migration/invasion and tube formation. The inhibitory action of endostatin has been attributed to its binding to the α5β1 integrin receptor (8) and possibly to its low-affinity binding to glypican-1 and -4 or its high-affinity binding to an unidentified molecule on endothelial cells (9). Blockage of *VEGF*/*VEGFR* signaling (10,11), inhibition of metalloproteinases, e.g., MMP-2 (12), and down-regulation of c-*MYC* and *cyclin D1* (13,14), are examples of the mechanisms through which endostatin signaling may lead to reduced endothelial cell survival, motility and invasion. A number of physiological functions of endostatin have been identified. The endostatin levels are elevated in certain types of cancer and chronic inflammatory diseases, e.g., rheumatoid arthritis (15) and diabetic retinopathy (16). Platelets were shown to sequester endostatin (17) for later release, e.g., to modulate wound healing. Endostatin also suppresses vascular permeability (18).

The mechanism of action of angiogenesis inhibitors on endothelial cells and their receptors has not been elucidated. Studies with platelet factor 4 (19) and thrombospondin (20) indicated that their heparin-binding domains were involved, by competing with the angiogenic basic fibroblast growth factor for binding to proteoglycan receptors. A strong affinity for heparin was also demonstrated for angiostatin and endostatin (21,22). However, other studies reported an inhibitory action of non-heparin-binding thrombospondin fragments (23,24), which may bind to the CD36 receptor (25), suggesting a complex array of biologically active sites. A similar complexity apparently exists for angiostatin, in which four individual kringle domains each exhibit antiproliferative activity, although to different extents (26). There was apparently no correlation with lysine affinity; however, whether there is a correlation with heparin affinity remains to be established. The diversity of angiogenesis inhibitors suggests that each component may require its own precise molecular

analysis and it is possible that no common interaction mechanism exists.

The specific functional site of endostatin has not yet been determined. Certain investigators (27) have constructed two mutants of endostatin, EM1 (9 amino residues of C-terminal were deleted) and EM2 (17 amino residues of C-terminal were deleted). EM1 and EM2 were administered to a renal cell carcinoma tumor xenograft model. EM1 retained the natural biological activity of endostatin, whereas EM2 exhibited loss of function. The results obtained indicated that C-terminal conservation may be crucial for the biological activity of endostatin.

To determine the function of endostatin in ascites hepatoma cells, we constructed a mutant of endostatin designated as EM13, by deleting 13 amino residues (LCIENSFMTSFSK) from its C-terminal. Plasmids that encode EM13 and wild-type endostatin, were then transfected into H22 ascites hepatoma cells. Transfected cells were implanted into nude mice and the resulting tumors were measured and examined. Based on those results we aimed to determine the function of endostatin and whether the 13 amino residues of its C-terminal are indispensable to its biological function.

Materials and methods

Plasmids and materials. Plasmid pEGFP-N2 was a kind gift from Professor Jianing Zhang, Dalian Medical University. pMD-18-T vector was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The RNA LA PCR™ kit (AMV) Ver. 2.1, the PCR Agarose Gel DNA Purification kit, the MiniBEST Plasmid Purification kit Ver. 2.0, *Xho*I and *Sac*II restriction enzymes, T4 DNA ligase, DL2000 DNA Marker, λ-*Hin*dIII, X-gal and IPTG were also purchased from Takara Biotechnology Co., Ltd. DEPC, RPMI-1640, G418 and Lipofectamine 2000 Transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA).

Construction of pEGFP-N2-endostatin and pEGFP-N2-EM13. The cDNA of endostatin was amplified from plasmid pBV220-endostatin by F01 and R02 primers. The cDNA of EM13 (amino acids 1-171), was amplified from mouse liver tissue by RT-PCR with the primers 5'-CTGCT CGAGATGCATACTCATCAGGACTT-3' and 5'-TAACCGC GGGACGATGTAGCTGTTGTGGC-3'. The product was incorporated into the pMD-18-T vector. After the plasmid was sequenced, the result revealed that the EM13 sequence contained in pMD18-T-EM13 was identical to the endostatin sequence (1-513) in the GenBank database.

To construct pEGFP-N2-endostatin (full-length), the PCR product of endostatin was excised (*Xho*I and *Sac*II). It was then inserted into an *Xho*I/*Sac*II site of the pEGFP-N2 vector. Following construction of pEGFP-N2-EM13 (amino acids 1-171), the cDNA of EM13 was excised from pMD-18-T-EM13 (*Xho*I and *Sac*II) and inserted into the pEGFP-N2 vector (*Xho*I/*Sac*II).

Cell culture, transfection and creation of stable cell lines. H22 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml L-glutamine in a humidified incubator at 37˚C with 5% CO_2. H22 cells were seeded at a density of $1x10^6$ cells/60-mm dish and transfected with pEGFP-N2-endostatin, pEGFP-N2-EM13 and pEGFP-N2 vector with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After being transfected for 24 h, H22 cells were determined by fluorescence microscopy and PCR to grow into stable cell lines and the transfected cells were screened in a selection medium containing 600 μg/ml G418. The screened cells were then cultured with G418 at a sustained concentration of 200 μg/ml.

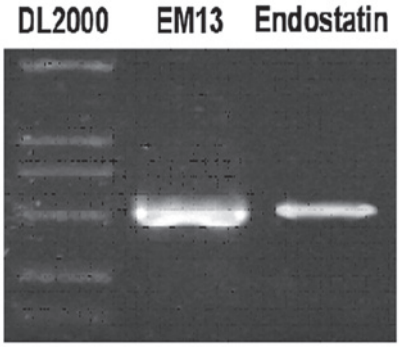

Figure 1. Extraction of total RNA and amplification of EM13. PCR amplification of EM13 gene: Lane 1, DL2000 DNA marker; lane 2, EM13; lane 3, endostatin.

Antitumor effect in vivo. Nude BALB/c mice, weighing 18-25 g, were used for the studies of endostatin on tumor growth *in vivo*. Six mice were injected subcutaneously with H22 cells stably transfected with pEGFP-N2-endostatin, pEGFP-N2-EM13 or vector control. After 16 days, the tumor weight was quantified. The density of blood vessels in each tumor tissue was examined under a light microscope following hematoxylin and eosin (H&E) staining. The blood vessel densities of each group were measured.

Results

Extraction of total RNA and amplification of EM13. Total RNA was extracted by TRIzol reagent from the mouse liver. Following extraction, the RNA was resolved into 30 μl ddH_2O. According to the endostatin sequence in GenBank, two primers were designed to amplify the full sequence of endostatin, except for the 13 aminos in the C-terminal. The recognition sites of *Xho*I and *Sac*II were introduced into the upstream (R01: 5'-CTGCTCGAGATGCATACTCATCAG GACTT-3') and downstream (F01: 5'-TAACCGCGGGACGATGTAGCTGTT GTGGC-3') primers, respectively. The product of RT-PCR with these primers was designated as EM13. The cDNA of endostatin was also amplified by PCR with primers F01 and R02 (Fig. 1), with pBV220-endostatin plasmid as the template.

Construction of expressing plasmid. The PCR products were incorporated into vector pMD18-T following purification. Clones with plasmid pMD18-T-EM13 were verified by PCR with primers F01 and R01 (Fig. 2A). One clone was selected and cultured and plasmid pMD18-T-EM13 was harvested (Fig. 2B). The plasmid was sequenced and the results demonstrated that

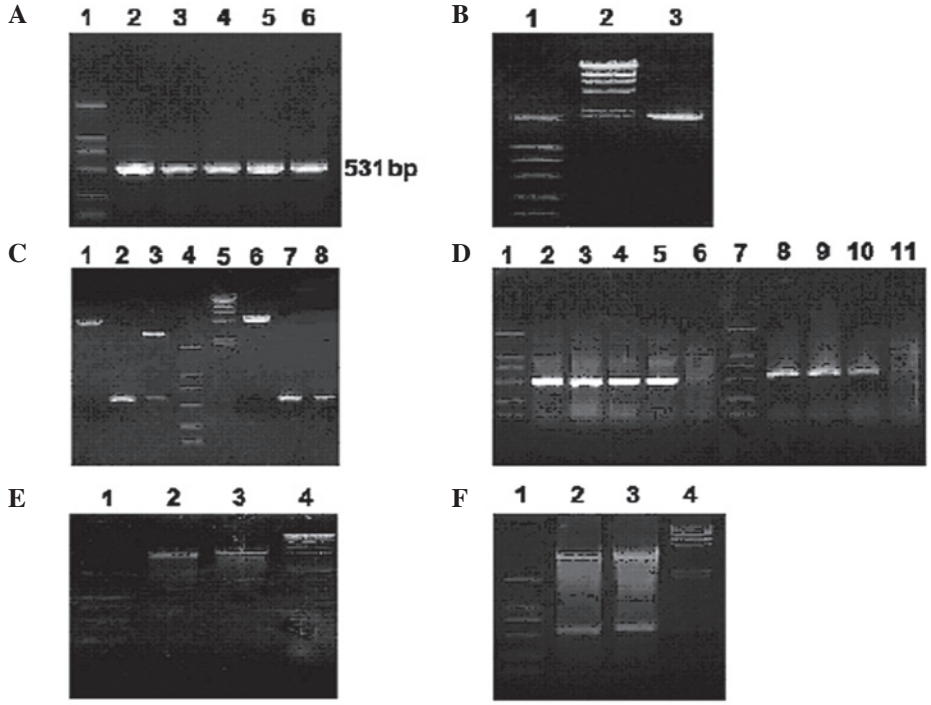

Figure 2. (A) Construction of expressing plasmid. Screening for endostatin 13-T-positive clones by polymerase chain reaction (PCR): Lane 1, DL2000 DNA marker; Lanes 2-6, PCR products no. 1-5. (B) Screening for endostatin 13-T-positive clones by plasmid extracting: Lane 1, DL2000 DNA marker; lane 2, λ-*Hin*dIII DNA marker; and lane 3, plasmid of positive clone pMD18-T-EM13. (C) Digestion of endostatin 13-T, endostatin, pEGFP-N2 by *Sac*II/*Xho*I: Lane 1, vector pEGFP-N2 digested by *Sac*II/*Xho*I; lane 2, endostatin digested by *Sac*II/*Xho*I; lane 3, pMD18-T-EM13 digested by *Sac*II/*Xho*I; lane 4, DL2000 DNA marker; lane 5, λ-*Hin*dIII DNA marker; lane 6, purification of pMD18-T-EM13 digestion product; lane 7, purification of endostatin digestion product; and lane 8, purification of pEGFP-N2 digestion product. (D) Screening for positive clones by PCR: Lane 1, DL2000 DNA marker; lane 2-5, PCR products no. 11-14; lanes 6 and 11, negative control of respective PCR; lanes 8-10, PCR products no. 21-23. (E) Plasmid pEGFP-N2-EM13 and pEGFP-N2-endostatin. Lane 1, DL2000 DNA marker; lane 2, pEGFP-N2-EM13; lane 3, pEGFP-N2-endostatin; and lane 4, λ-*Hin*dIII DNA marker. (F) Plasmid pEGFP-N2-EM13 and pEGFP-N2-endostatin were digested with *Xho*I and *Sac*II. Lane 1, DL2000 DNA marker; lane 2, pEGFP-N2-EM13 was digested with *Xho*I and *Sac*II; lane 3, pEGFP-N2-endostatin was digested with *Xho*I and *Sac*II; and lane 4, λ-*Hin*dIII DNA marker.

the endostatin sequence contained in pMD18-T-EM13 was identical to the endostatin sequence in GenBank.

The plasmid pMD18-T-EM13 and the fragment of endostatin were digested with *Xho*I and *Sac*II and the fragment of endostatin and EM13 was inserted into the plasmid pEGFP-N2, which was also digested by *Xho*I and *Sac*II (Fig. 2C). Through PCR screening, 4 clones possibly carrying pEGFP-N2-endostatin and 3 clones possibly carrying pEGFP-N2-EM13 were selected (Fig. 2D). One clone from each group was selected for harvesting plasmids (Fig. 2E). The two plasmids were also verified by digestion with *Xho*I and *Sac*II (Fig. 2F).

Endostatin inhibits tumor growth and blood vessel formation. Plasmids pEGFP-N2-endostatin and pEGFP-N2-EM13 were transfected into H22 cells. After 36 h the efficiency of transfection was examined under a fluorescent microscope (the transfected cells exhibited green fluorescence). To obtain stable cell lines, transfected cells were screened in a selection medium containing 600 μg/ml G418 and the screened cells were then cultured with G418 at a sustained concentration of 200 μg/ml.

To determine whether endostatin is able to suppress tumorigenesis and the formation of capillaries, H22 cells and H22 cells constantly transfected with plasmids pEGFP-N2-endostatin or pEGFP-N2-EM13 were implanted into BALB/c mice (six mice/group). Sixteen days later, the mice were sacrificed, the tumors were weighed and the density of blood vessels was also measured by H&E staining. Compared to the untransfected group, the tumor growth of the group transfected with endostatin was relatively slow and exhibited a prolonged incubation period. However, the result did not differ significantly between the untransfected group and the group transfected with endostatin. The tumor weight of the group transfected with EM13, compared to the untransfected group, exhibited no statistically significant difference (Table I). Following H&E staining, the histological examination revealed a relatively low density of tumor capillaries in the group transfected with endostatin compared to the untransfected group, while the group transfected with EM13 exhibited no significant difference compared to the untransfected group (Fig. 3).

Table I. Inhibitory effect of endostatin on tumor growth.

Groups	Tumor weight (g)	P-value
Untransfected	1.2489±0.4166	-
PEGFP-N2-vector	1.0973±0.3472	0.510
PEGFP-N2-endostatin	0.8914±0.1027	0.091
PEGFP-N2-EM13	1.2005±0.3736	0.837

Our results have demonstrated that endostatin may play an important role in inhibiting tumor growth and the formation

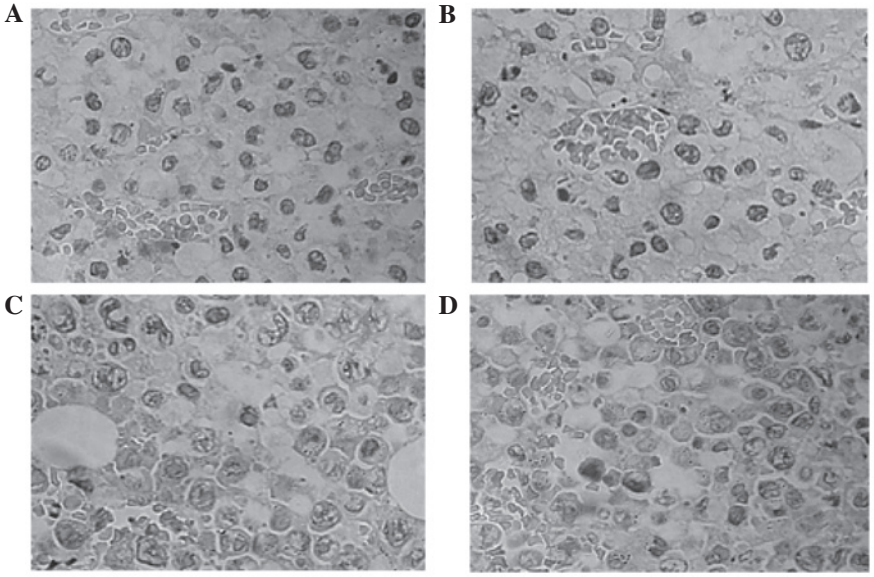

Figure 3. Endostatin inhibits tumor growth and blood vessel formation. After mice were sacrificed, the tumor density of blood vessels was observed under a light microscope following hematoxylin and eosin staining. (A) Untransfected, (B) transfected with pEGFP-N2-vector, (C) transfected with pEGFP-N2-endostatin and (D) transfected with pEGFP-N2-EM13.

of blood vessels and that the 13 amino acids at the C-terminal may be a region indispensable to the biological activity of endostatin.

Discussion

Angiogenesis is the sprouting of capillaries from preexisting blood vessels by the proliferation, differentiation and migration of endothelial cells. This physiological process is closely regulated by a delicate balance between pro- and anti-angiogenic factors. An imbalance of the angiogenic process contributes to the development of a number of disorders. It is well established that angiogenesis is vital for the development, progression and metastasis of a number of human solid tumors.

The growth and progression of solid tumors beyond 2 mm^3 is dependent on the recruitment of angiogenic vessels and an expansion of the tumor vasculature. Investigations have been mainly focused on the inhibitors of angiogenesis that are required for tumor growth and metastases. Previous studies (10-14) have clearly defined some of the angiogenic factors that contribute to tumor growth. In this study, the potential antitumorigenic activity of endostatin and EM13 in the H22 mouse hepatocellular carcinoma model was investigated.

The plasmids expressing endostatin and EM13 gene were transfected into hepatoma H22 cells with the cationic liposome-mediated method. Following G418 screening, the cells were subcutaneously inoculated in BALB/c inbred mice. Our data have demonstrated that there was no significant difference in tumor weight between the control and transfected groups. The H&E staining demonstrated that the group transfected with endostatin exhibited a relatively low density of tumor capillaries compared to the control group; however, the group transfected with EM13 exhibited the same result as the untransfected group.

The results suggested that the group transfected with endostatin exhibited tumor growth inhibition to a certain extent. The tumor cell types determine the difference of the sensitivity of endostatin. The relatively low quantity of blood vessels in the ascitic hepatoma may have no effect on tumor weight between the untransfected and endostatin groups (Table I).

Cancer cells produce various vascular growth factors which may induce the host blood vessels to grow into the tumor and ensure nutrition supply. The growth velocity and biological characteristics of the tumor are associated with angiogenesis and different types of tumors exhibit different intensities of angiogenic activity. Tumor cells that arise from ascites hepatoma and HL60 cells (a leukemia cell line) do not possess angiogenic activity. However, following inoculation of Ehrlich ascites cells into experimental animals and the formation of a solid tumor, the angiogenic activity reappears, indicating that angiogenic activity is closely associated with tumor type and living environment. A possible mechanism explaining this phenomenon is that it may be easy for the floating cells to uptake nutrients from a liquid environment. Due to the ascites tumor having a relatively low density of blood vessels, endostatin exerts almost no effect.

In a previous study by Peroulis *et al* (28), C6 glioma cells transfected with endostatin by the cationic liposome-mediated method were subcutaneously inoculated into rats. The expression of endostatin detected by RT-PCR and western blot analysis was low and the tumor inhibitory effect was not significant. Although a number of studies reported that endostatin treatment reduced tumor growth rates and induced the regression of established tumors (2,29-31), complete tumor inhibition was not readily achieved.

The assessment of the endostatin and EM13 treatment models demonstrated that endostatin therapy alone may not be sufficient for the complete regression of all types of tumors. The persistent tumor growth and the variations in the extent

of regression suggest that the targeting of tumor angiogenesis alone may not effectively treat all tumors. Therefore, endostatin treatment administered in combination with chemo- or immunotherapy may lead to tumor growth arrest and a significantly reduced tumor growth rate.

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