Antitumor effect of endostatin overexpressed in C6 glioma cells is associated with the down-regulation of VEGF

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Abstract. Endostatin is an anti-angiogenic agent that blocks endothelial cell proliferation, tumor growth, and metastasis. Several lines of direct evidence show that gliomas express high levels of endostatin. However, the anti-angiogenic activity and cellular mechanisms of endostatin from tumor cells have not been completely elucidated. In this study, we established C6 glioblastoma (GBM) xenografts in nude mice by subcutaneously injecting glioma cell lines, C6-null cells, and stable transfected-C6 cells overexpressing mock vector (C6-mock) and endostatin (C6-endo). We found that overexpression of endostatin in C6-endo cells significantly suppressed the expression of VEGF in tumor cells *in vivo* as well as *in vitro*. Furthermore, the tumor growth derived from C6-endo cells was inhibited. Our data demonstrate that endostatin could play a direct role in inhibiting tumor cells, and suggest that enhancing endostatin expression in gliomas could be an effective treatment strategy.

Introduction

Glioblastoma multiforme (GBM) is characterized by neovascularization, raising the question of whether angiogenic inhibition may be a useful therapeutic strategy for this tumor. Several studies have shown that angiogenesis is essential for tumor growth and metastases, particularly in GBM (1,2). It has been suggested that the onset of angiogenesis is one key step during brain tumor progression, and angiogenic inhibition may be sufficient in overcoming proangiogenic signals from tumor cells (3). To stimulate angiogenesis, tumors produce angiogenic factors that induce endothelial proliferation, assembling a new vascular network that facilities tumor expansion and can predict prognosis. During neovascularization, tumor cells generate a variety of molecules that initiate or inhibit angiogenesis, suggesting that the angiogenic phenotype is the result of a balance between endogenous positive and negative regulation of neovascularization (1,3,5).

Among the inhibitors of angiogenesis, several are endogenous factors that have been shown to affect glioma growth *in vivo* (5-8). Endostatin was initially derived from a C-terminal proteolytic fragment of type XVIII collagen, an extracellular matrix component in blood vessel walls (5,9) that has been shown to be an endogenous inhibitor of endothelial cell proliferation, angiogenesis and tumor growth (5-7,9-12). Several lines of direct evidence showed that gliomas express high levels of endostatin (13,14). However, the anti-angiogenic activity of endostatin from tumor cells have not been completely elucidated. In this pilot study, we used genetically modified tumor cells that stably expressed endostatin to establish *in vivo* xenograft glioma tumor models. These models provide a local delivery of endostatin from tumor cells that allowed us to assess *in vivo* biological efficacy and investigate its cellular mechanisms.

Materials and methods

Plasmids. Full-length rat endostatin cDNA was amplified based on sequence provided by Genebank (GenBank accession no.: NM_009928). Primers were designed using Primer Express® 1.5a software and were as follows: Primer 1 (open reading frame 'ORF') F: 5'-TTACCCGGGAGTTCCACA CC-3', R: 5'-TGTGTCAAAGTTCTGCATCGC-3'; Primer 2, F with sequence of signal peptide Igκ and marker protein HA, F: 5'-GAATCACAGACCCAGGTCCTCATGTCCCTG CTGCTCTGGATTTCTGGTACCTGTGGGTATCCATAT

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Abbreviations: VEGF, vascular endothelial growth factor; GBM, glioblastoma; HUVEC, human umbilical vein endothelial cells; MVD, microvessel density

Key words: endostatin, vascular endothelial growth factor, angiogenesis, anti-angiogenic agent, glioma

GATGTTCCAGATTATGCTGGCCATACTCACCAGGAC TTTC-3', R: 5'CCGCTCGAGCTATTTGGAGAAAGAG GTC-3'; Primer 3, F with sequence of restriction sites and initial codon 5'-GGGGTACCATGGAATCACAGACCCAG GTCCTC-3', R: with sequence of restriction sites and stop codon, 5'-CCGCTCGAGCTATTTGGAGAAAGAGGTC-3'. The amplicon with *Kpn*I-*Xho*I digestion was cloned into pBudCE4.1 expression vector (Invitrogen, Carlsbad, CA, USA) containing HA and V_5 tag under the control of human pEF-1α promoter to generate plasmid pBudCE4.1/endo.

Cell culture. Rat glioma C6 cells (Cell Biology Research Institute of Shanghai, Shanghai, China) were cultured in RPMI-1640 medium (1640M, Invitrogen) supplemented with fetal calf serum (FCS, 10%). A total of $2x10^5$ C6 cells were placed in 35-mm dishes and transfected with plasmids pBudCE4.1/endo (1 μg) or vector control pBudCE4.1 (1 μg) by using Lipofectamine™ 2000 (Invitrogen). Medium was replaced with 1640M containing Zeocin (1 mg/ml, Invitrogen) 18 h post-transfection. After 4 weeks, surviving clones were isolated, analyzed by PCR, and selected for a transfected cell line that demonstrated the highest expression of genes to generate heterogeneously overexpressing endostatin (C6-endo) and vector control (C6-mock).

For cell proliferation assay, $2x10^4$ cells were placed in 6-well plates and counted by a hemocytometer. For immunocytochemistry analysis, cells were first seeded on glass slides in a culture dish and cultured as previously described for 12 h, washed twice with RPMI-1640M, and cultured in serum-free RPMI 1640M for 24 h. For each cell line, 5 culture slides were washed and fixed for immunohistochemistry.

Endostatin activity assay in vitro. C6 cells, C6-mock cells, and C6-endo cells were plated onto 75-mm plates at 5-10 million cells/plate and incubated for 48-72 h. Between 80-90% confluence of cells were washed with PBS, and 5 ml of 1640M were added to each plate. Cells were incubated for an additional 24 h, and 5 ml of conditioned serum-free medium was collected and concentrated to 0.4 ml using a Centriplus 10 concentrator. Human umbilical vein endothelial cells (HUVEC) (Cell Biology Research Institute of Shanghai) were plated at 12,500 cells/well in 24-well collagen-coated plates for 24 h, after which 250 μl of concentrated, conditioned medium were added to each well for 30 min. An equal volume of 1640M supplemented with 10% fetal bovine serum was then added for an additional 72 h of incubation. The number of cells was then quantified using a colorimetric MTT assay. Tests were performed in quadruplicate.

Quantitative polymerase chain reaction. Total RNA was isolated and cDNA was synthesized as described previously (15). The sequences of primer sets were: VEGF, forward: CCCAAGCTTATGAACTTTCTGCTCTCTTG, reverse: CGCGGATCCTCACCGCCTTGGCTTGTC; ß-actin, forward: GAGGCATCCTGACCCTGAAG, reverse: CATC ACAATGCCAGTGGTACG. The calculation of expression levels of VEGF was normalized by ß-actin.

Immunohistochemistry. To detect VEGF and endostatin expression *in vitro*, cells were fixed in 4% paraformaldehyde and blocked with 3% normal goat serum for 2 h at room temperature for immunocytochemistry analysis.

To determine VEGF and endostatin expression *in vivo*, anesthetized mice were decapitated, and tumor tissues were removed and quickly fixed in 10% formalin. For immunohistochemistry, 4-μm-thick sections were cut and rehydrated, treated with 0.3% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase, rinsed with 0.1 M phosphate buffer (PB) for 10 min, and exposed to blocking serum (3% normal goat serum) for 2 h at room temperature.

Immunoreactions were performed as previously described (15). After incubation with anti-VEGF (1:150 dilution, United States Biological, Swampscott, MA, USA), anti-HA (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-endostatin (1:100, Oncogene, MA, USA), the slices were rinsed with 0.1 M PB and exposed to anti-rabbit IgG HRP (1:500, Maixin, Fuzhou, China). After an additional 10-min rinse, the slices were treated with VectaStain® Elite ABC reagent (Maixin) for 30 min and developed with DAB detection kit (Maixin Group, China). The slices were counterstained by hematoxylin and mounted by Permount (Maixin).

Angiogenesis detection. Microvessel density (MVD) was determined by CD31 antigen (1:150 dilution, Santa Cruz Biotechnology), and stained with streptavidin-peroxidase (SP) conjugation kit (Maixin) following the manufacturer's directions. As previously described (16), the microvessels were carefully counted under a confocal microscope (Nikon, Japan) in 5 fields (x200) around the basal part of tumor tissues and recorded as the average value of microvessel density (MVD). Single cells, a cluster of cells, and branches of vascular trunks were all counted as a blood vessel if they were CD31-positive.

Enzyme-linked immunosorbent assay (ELISA). To measure VEGF secretion *in vitro*, $5x10^5$ cells were placed in 6-well plates and treated with serum-free 1640M medium. Medium was collected after 24 h and after 48 h of culture. Debris was removed by centrifugation at 2,000 x g for 5 min and the supernatant collected for ELISA assay.

To measure VEGF levels *in vivo*, tumor tissues (0.1 g) were homogenized in Tris-HCl buffer (25 mM, pH 7.6) containing 100 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Debris was removed by centrifugation at 2,000 x g for 5 min, followed by centrifugation at 20,000 x g for 20 min, and supernatants were collected for ELISA assay. Protein concentrations were measured using Protein assay kit (Bio-Rad, Hercules, CA, USA).

Series dilutions of samples with the highest and the lowest expected values were performed to determine VEGF expression level using commercial VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. VEGF expression levels were calculated by a standard curve available from R&D Systems. All experiments were performed in triplicate.

Protein analysis. To analyze endostatin secretion *in vitro* as described previously, conditioned medium (CM) was collected

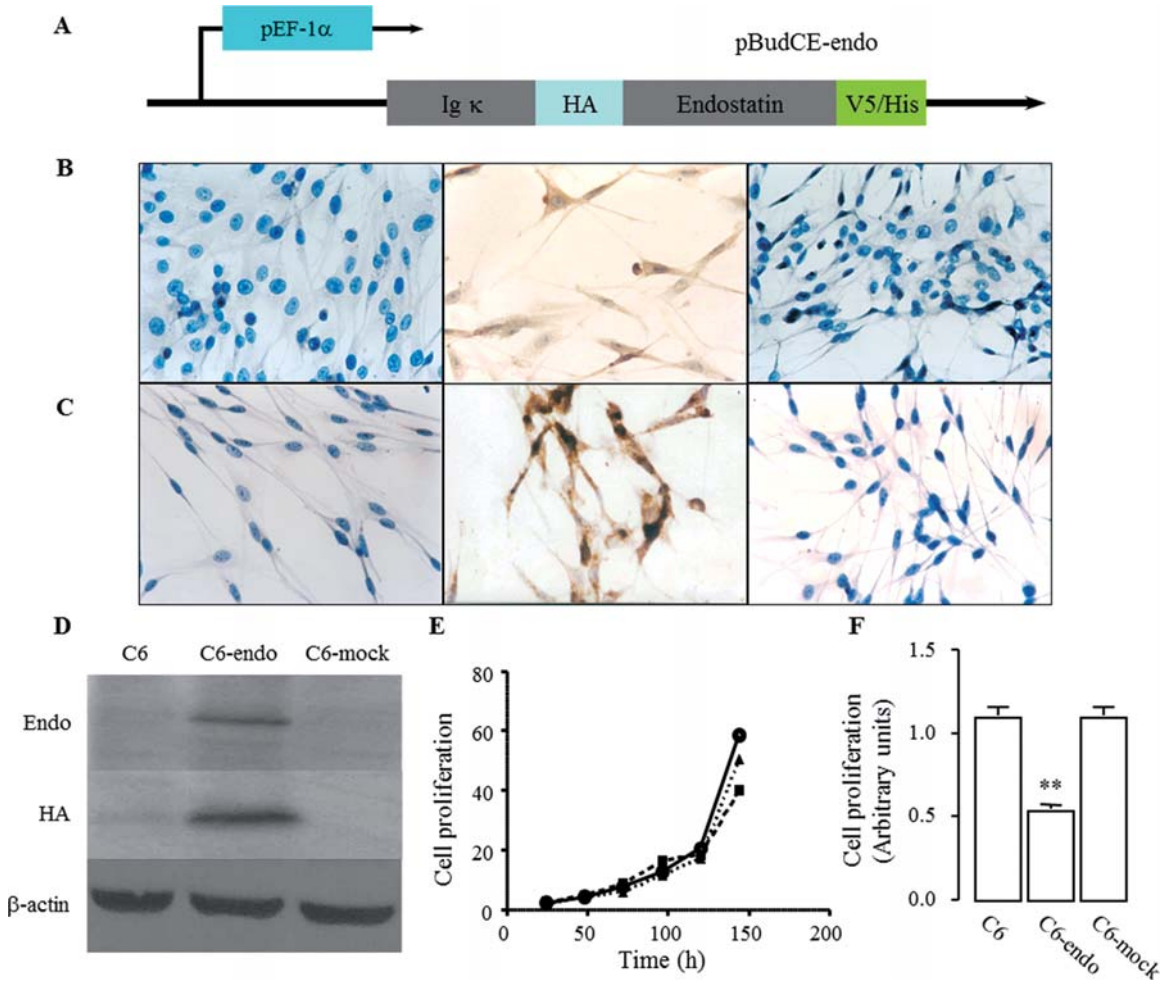

Figure 1. Characterization of overexpression of endostatin in C6 glioma cells. (A) The plasmid pBudCE4.1-endo encoding rat endostatin cDNA fused with HA tag and was controlled under human pEF-1α promoter. (B and C) Immunocytochemical staining with antibodies against endostatin (B) and HA (C) on C6 cells (left panel), and its heterogeneously expressing pBudCE4.1 vector (right panel) and pBudCE4.1-endo (middle panel). Bottom panel, detected by ß-actin antibody. (D) Western blot analyses of genetically modified C6 cells using antibodies against endostatin (upper panel) and HA (middle panel). Bottom panel, detected by ß-actin antibody. (E) Time course of cell proliferation of C6-null cells (open squares), C6-mock cells (closed squares), and C6-endo cells (closed circles). (F) MTT assay of HUVEC proliferation treated with culture media derived from C6-null cells, C6-mock cells, and C6-endo cells. $^{**}p<0.05$; n=4-6.

at 72 h postculture from individual clones and clarified by centrifugation at 800 x g for 10 min. Total proteins were concentrated by combining CM with 8 Vol of chilled 100% (V/V) methanol and incubated overnight at -20˚C. Samples were then centrifuged at 800 x g for 15 min at room temperature. The protein pellets were resuspended in PBS containing several standard protease inhibitors. CM was separated by SDS-PAGE, and endostatin was detected by Western blot analysis using an anti-HA antibody (1:250, Santa Cruz Biotechnology) and anti-endostatin antibody (1:100, Oncogene).

To analyze endostatin secretion *in vivo*, tumor tissues were homogenized in Tris-HCl buffer (50 mM, pH 8.0) containing the protease inhibitor cocktail V (Calbiochem, San Diego, CA, USA). Homogenate (20 μg) proteins were separated by electrophoresis on 4-20% SDS-page gel and transferred onto Immobilon membranes (Millipore, Billerica, MA, USA). Western blot analyses were conducted using antibodies against endostatin (1:100), HA (1:250), and ß-actin (1:2000, Neomarker, Fremont, CA, USA). Bands were visualized using an electrochemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ, USA).

Xenograft glioma animals. Male BALB/c (*nu/nu*) 4-6 week-old mice were purchased from Charles River (Shanghai, China) and were subcutaneously injected into the flank with $1.5x10^6$ cells in serum-free 1640M. Twenty days post-implantation, animals were sacrificed and the tumors removed for rapid freezing in liquid nitrogen. All procedures met the national guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the Fujian Medical University, Fujian, China.

Statistical analysis. Data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test for multiple comparisons to control groups. Differences were considered significant at $p<0.05$.

Results

Effect of endostatin from glioma cells in vascular endothelial cell proliferation in vitro. We cloned rat endostatin cDNA into pBudCE vector-contained Igκ signal that released endostatin into the culture medium (Fig. 1A). First, we verified the expression of endostatin in genetically modified by immuno-

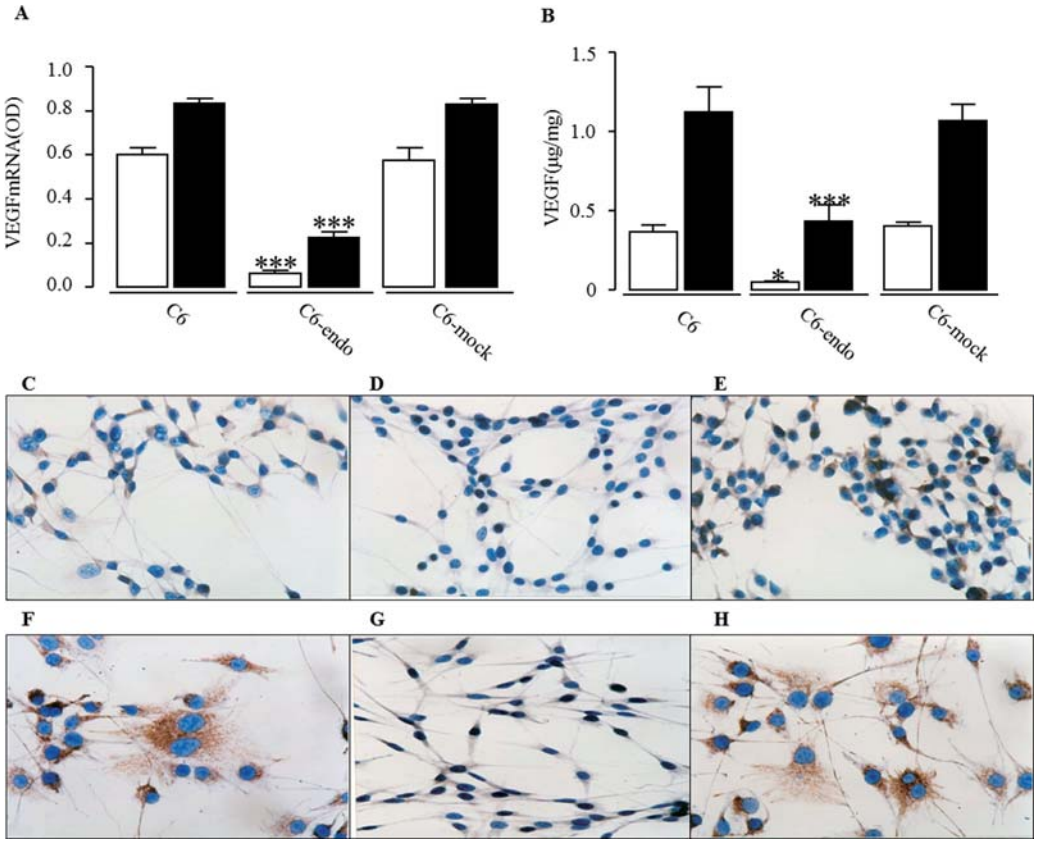

Figure 2. The levels of VEGF, detected by RT-PCR (A) and ELISA (B) on glioma cells, after 24 h (open bars) and 48 h (closed bars). $^{*}p<0.05$; $^{***}p<0.001$; n=4-6. Immunocytochemical staining with antibody against VEGF antigen in C6-null cells (C and F), C6-endo cells (D and G), and C6-mock (E and H) after 24 h (C-E) and 48 h (F-H) serum deprivation.

staining and Western blot analysis (Fig. 1B-D). Immunoreaction with antibodies against endostatin (Fig. 1B) or HA (Fig. 1C) showed that rat C6 cells heterogeneous overexpression of endostatin (C6-endo, middle panels) was higher compared with C6-null cells (left panels) and C6-mock cells (right panels), as well as that detected by Western blotting (Fig. 1D). Endostatin expression did not significantly affect C6 cell proliferation (Fig. 1E). Studies have suggested that endostatin is an inhibitor of endothelial cell proliferation (12). We treated HUVEC with the culture media harvested from C6-null cells, C6-endo cells, and C6-mock cells. Our findings showed that culture media from C6-endo cells suppressed HUVEC growth compared with C6-null or C6-endo cells (Fig. 1F), suggesting that the endostatin from glioma cells inhibits vascular endothelial cell proliferation.

Endostatin antagonizes VEGF expression levels in vitro. We verified VEGF expression levels in the heterogeneously overexpressing endostatin cells by RT-PCR, ELISA assay, and immunostaining. We found that VEGF expression levels of mRNA (Fig. 2A) were significantly lower in C6-endo cells compared with expression levels in C6-null cells or C6-mock cells. Simultaneously, we determined VEGF protein secretion in those cells and found that the levels of VEGF in medium from C6-endo cells were significantly lower than VEGF levels from C6-null cells and C6-mock cells (Fig. 2B). These changes were observed similarly after 24 and 48 h of serum deprivation (Fig. 2B). In addition, immunoreaction with antibody against VEGF showed that endogenous VEGF

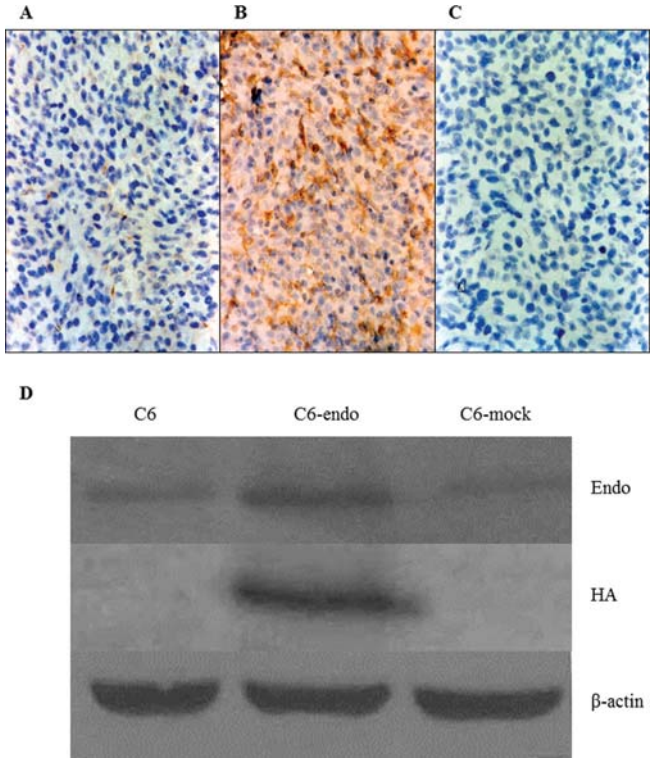

Figure 3. Immunohistochemistry staining with antibodies against HA on xenograft glioma tumors derived from C6 null cells (A), C6-endo cells (B), and C6-mock cells (C). (D) Western blot analyses of genetically modified rat C6 cells using antibodies against endostatin (upper panel) and HA (middle panel).

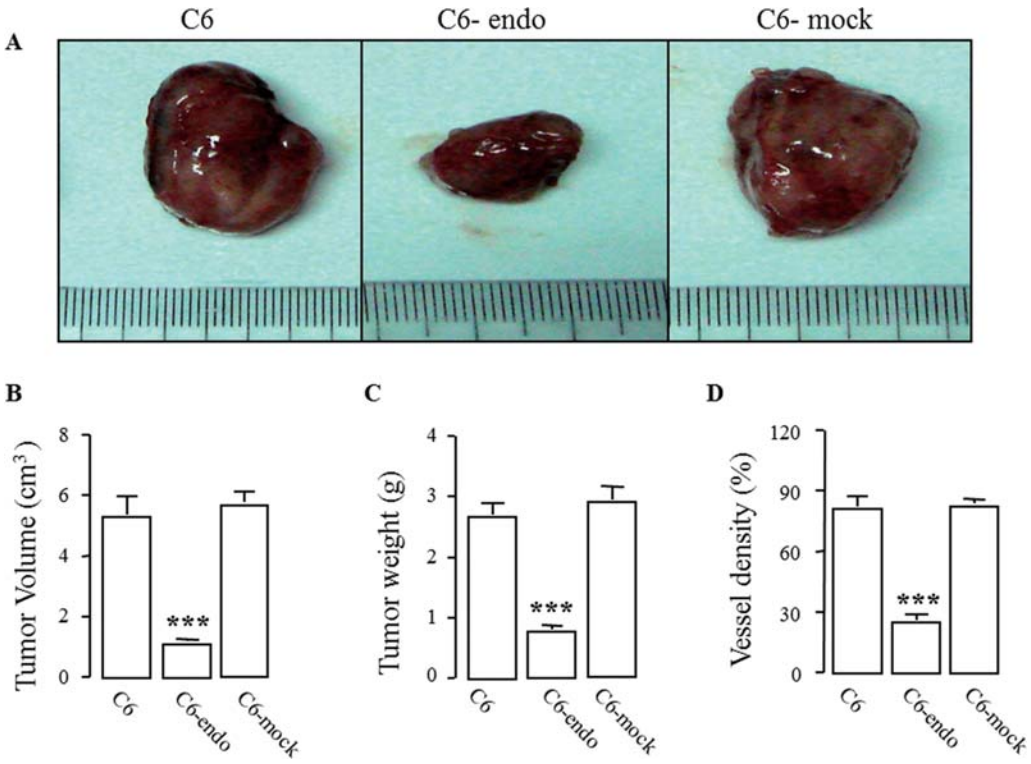

Figure 4. Xenograft glioma tumor were generated by subcutaneous implantation of C6 null cells, C6-endo cells and C6-mock cells. (A) Tumors removed from mice after 20-days implantation. Characterization of xenograft glioma tumors by the size (B), weight (C), and vessel density (D). ***p<0.01; n=7.

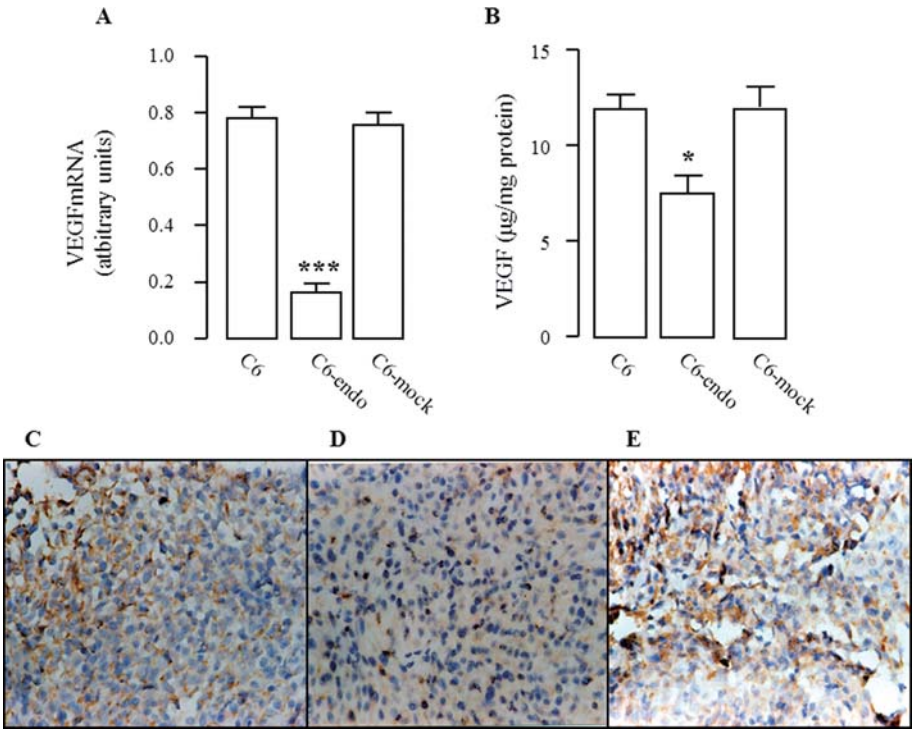

Figure 5. The levels of VEGF, detected by RT-PCR (A) and ELISA analysis (B), on xenograft glioma tumors. *p<0.05; ***p<0.001, n=5. Immunohistochemistry staining with antibodies against VEGF on xenograft glioma tumors derived from C6-null cells (C), C6-endo cells (D), and C6-mock cells (E).

expression was reduced in C6-endo cells compared with C6-null cells and C6-mock cells after 24 h of serum deprivation (Fig. 2C-E) and 48 h of serum deprivation (Fig. 2F-H). This shows that endostatin directly antagonizes VEGF expression in glioma tumor cells.

Xenograft glioma tumor cells express endostatin. To confirm the expression of endostatin *in vivo* and *in vitro*, we determined endostatin levels in genetically modified rat C6 cells by immunostaining and Western blot analysis (Fig. 3). Immunoreaction with antibodies against HA showed that expression

levels of endostatin in tumors from C6-endo cells (Fig. 3B) appeared higher compared with C6-null cells (Fig. 3A) and C6-mock cells (Fig. 3C), as well as that detected by Western blot analysis (Fig. 3D). These data suggest that xenograft glioma tumor cells express endostatin.

Endostatin suppresses tumor growth. Endostatin has been shown to inhibit endothelial cell proliferation, angiogenesis, and tumor growth (9,12). To evaluate the anti-tumor potential of endostatin, we injected C6-null cells, C6-endo cells, and C6-mock cells directly into the mouse right inguinal area to induce tumor formation. After 20 days, tumors derived from C6-endo cells were noticeably smaller compared with tumors from C6 null cells and C6-mock cells (Fig. 4A). The tumor sizes and weights of xenograft mice are shown in Fig. 4B and C.

Endostatin decreases the vascular formation in vivo. We tested whether endostatin-supressed tumorigenesis is mediated by its anti-angiogenesis properties. Immunohistochemistry analysis showed that vascular formation (Fig. 4D) in tumor tissues was markedly reduced in the tumors derived from C6-endo cells relative to vacularization in the tumors derived from C6 cells and C6-mock cells, indicating that tumor-induced angiogenesis was inhibited by endostatin.

Endostatin antagonizes VEGF expression levels in vivo. Studies have shown that VEGF plays a role in tumorigenesis (17,18), and that targeting the VEGF pathway is an effective anti-tumor therapy. To understand the role of VEGF on endostatin-supressed tumorigenesis, we examined the VEGF expression levels on glioma xenograft tumors. We found that VEGF expression levels of mRNA (Fig. 5A) and protein (Fig. 5B) were significantly lower in tumors derived from C6-endo cells compared with the levels from C6-null cells or C6-mock cells. Similar results were further confirmed by immunohistochemistry using antibodies against VEGF (Fig. 5). Fig. 5 shows that the expression of VEGF was markedly lower in tissue sections of glioma xenograft mice derived from C6-endo cells (Fig. 5D) compared with the expression from C6-null cells (Fig. 5C) and C6-mock cells (Fig. 5E). This suggests that endostatin antagonizes VEGF expression levels, in turn inhibiting tumorigenesis.

Discussion

The main finding of this study demonstrated that local delivery of endostatin from tumor cells suppressed tumorigenesis and endothelial cell proliferation. This anti-tumor effect is most likely a result of anti-angiogenic activity through the inhibition of VEGF expression in tumor cells.

The anti-tumor potency of endostatin has been controversial over the last decade. Initial reports demonstrated regression of pre-established subcutaneous tumors in mice that failed to regrow after repeated endostatin regimen (9,19). Although most subsequent studies confirmed endostatin's inhibitory effect on tumor growth (7,20), additional contradictory results reported that endostatin had no effect on tumor growth when high systemic concentrations were administrated (21). Notably, high systemic levels of endostatin may not necessarily produce sufficient concentrations at the tumor site. Therefore, our genetic modification of rat glioblastoma C6 cells that stably released endostatin provided local delivery, eliminated variations in biological distribution, allowing us to directly evaluate *in vivo* biological efficacy of endostatin. In this study, our data are in line with other experimental studies in which endostatin suppress tumor growth (19,20,22-24).

Vascularization of malignant gliomas requires increased angiogenesis, which depends on endothelial cell proliferation (25). Despite studies that have shown that endostatin inhibits tumors, our *in vitro* results ruled out a direct antiproliferative effect on vascular endothelial cells that pointed to the anti-angiogenic effect of endostatin as main anti-tumor mechanism. Although we did not study the molecular mechanism for the inhibition of angiogenesis, the histologic data obtained from xenograft tumor tissues showed that endostatin inhibits vessel formation. Under physiological conditions, angiogenesis is regulated by a balance between stimulatory and inhibitory modulators. Although several lines of direct evidence showed that gliomas express high levels of endostatin, these phenomena seem to be a result of large amounts of VEGF secreted from malignant glioma cells (13,14). Thus, a net balance between angiogenic stimulators and inhibitors may be tipped toward angiogenesis (14). In our models, the contribution of endostatin to tumor growth and vascularization was dependent on the expression levels of tumor cell-derived endostatin. Compared with C6 mice or C6-mock mice, neovascularization was markedly decreased in C6-endo mice, the result is consistent with that of Yamaguchi *et al* (26).

Although it is known that endostatin can inhibit VEGF-induced endothelial cell migration and proliferation (27,28), our results clearly demonstrate that endostatin plays a direct role in tumor cells, the endostatin anti-angiogenic actions are mediated by down-regulating VEGF expression in glioma cells. *In vitro*, VEGF concentrations in the culture media of C6-endo cells were lower than that of C6-mock cells and C6 cells. In our study, we also simultaneously assessed the expression levels of VEGF mRNA and protein in xenograft glioma mice derived from genetically modified C6 cells. These studies showed that VEGF expression were lower in the tumors derived from C6-endo cells relative to levels in the tumors derived from C6 cell and C6-mock cells. These data indicate that the reduction of tumor growth and angiogenesis induced by endostatin from tumor cells was a result of VEGF down-regulation in tumor cells. Although more work is needed to clarify how endostatin down-regulates VEGF expression in tumor cells, this pilot study provides insight into how endostatin plays a direct role in tumor VEGF expression. If we assume that endostatin is a negative feedback product of VEGF, then the higher the endostatin levels, the lower the VEGF levels.

In conclusion, our data provide evidence that the endostatin activity could be extended from its action on endothelial cells to an effect on tumor cells. Endostatin may have multiple effects leading to anti-angiogenesis. First, endostatin directly inhibits endothelial cell proliferation (9,12). Second, as we demonstrated *in vitro* and *in vivo*, endostatin directly controls VEGF production in tumor cells to modulate angiogenesis and neovascularization. Together, these findings strongly suggest that the actions of endostatin

on tumor cells contribute, at least in part, to its anti-angiogenic potency, and enhancement of endostatin expression in tumor may be an effective strategy for treating gliomas. Clinical studies will be needed to verify this.

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