Inhibition of xenograft human glioma tumor growth by lentivirus-mediated gene transfer of alphastatin

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Abstract. Angiogenesis is crucial for the development and metastasis of human brain glioma. Based on our previous successful construction of a lentivirus-mediated alphastatin (an endogenous angiogenesis inhibitor) gene transfer system and our findings that alphastatin exhibited potent inhibitory effects on the migration and differentiation of human umbilical vein endothelial cell lines (HUVECs) induced by vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) *in vitro*, here, we investigated the effect of using lentiviral vectors to overexpress alphastatin in human glioma cells to show whether sustained long-term expression of alphastatin diminishes tumor growth in a xenograft glioma model. We found that the transduced glioma cells sustainedly secreted alphastatin, which did not affect the proliferative ability of the glioma cells. Furthermore, tumor xenografts treated with the recombinant lentivirus were significantly smaller compared to the control xenografts and vascularity within the treated tumors was evidently decreased. Our data suggest that stable expression of alphastatin inhibits human glioma growth by inhibiting angiogenesis, with a probable mechanism of suppressing the turnover of VE-cadherin membrane molecules.

Introduction

Malignant gliomas are among the most extensively vascularized human tumors (1,2), which is further supported by the identification of tumor blood vessel density as an independent prognostic parameter for human astroglial tumors (3). The prognosis of malignant gliomas is still dismal despite aggressive treatment attempts. Thus, alternative therapy strategies are needed. Since malignant gliomas are characterized by extensive vascularization and their proliferation is hallmarked by a distinct proliferative vascular component (3), it seems to be a logical consequence to apply antiangiogenic treatment strategies to malignant gliomas. Recently, a large, randomized phase II trail of bevacizumab, pan-VEGFR inhibitor cediranib and other angiogenesis inhibitors was completed (4,5). Preliminary results confirmed the safety of these agents and showed a significant increase in the rate of progress-free survival for patients with malignant gliomas.

There are several angiogenesis inhibitors in the body, which help to suppress pathologic angiogenesis (6-8). Alphastatin, an endogenous angiogenesis inhibitor, is a 24-amino acid peptide derived from the amino terminus of the α chain of human fibrinogen, and has potent antiangiogenic properties *in vitro* and in tumor models (9-11). In our previous detailed *in vitro* and *in vivo* study of angiogenesis inhibition, we utilized a lentivirus-mediated gene transfer system that allows local sustained long-term expression of alphastatin (11) and established models for the prevention of human glioma tumorigenesis. In this study, the recombinant alphastatin lentiviruses were able to stably infect human umbilical vein endothelial cell lines (HUVECs), and exhibited potent inhibitory effects on HUVEC migration and differentiation induced by vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). Notably, our data also showed that the stable expression of alphastatin in HUVECs evidently inhibited human glioma tumorigenicity by inhibiting angiogenesis. Furthermore, according to alterations in protein expression during HUVEC tube formation induced by the activation of mitogen-activated protein kinase (MAPK) signaling, we determined that alphastatin inhibited VEGF- or bFGF-induced angiogenesis by the blocking JNK and ERK phosphorylation pathway (11).

To assess the antitumor effects of secreted protein alphastatin and to extend these observations, in the present study, we chose to establish a treatment model of human glioma to assess whether alphastatin could be secreted in human glioma cells transduced by an alphastatin gene delivery system, and simultaneously evaluated the inhibition efficacy of tumor growth following treatment with the secreted protein alphastatin, in order to identify the antitumor mechanism of alphastatin. We

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report here that the lentivirus-mediated gene delivery system of alphastatin is an effective method for the treatment of human malignant glioma of the brain.

Materials and methods

Cells and reagents. HUVECs, and human glioma cell lines SHG44 and U87 were purchased from Keygen (Yuhuatai, Nanjing, China). Cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin and 1% streptomycin, and were grown at 37˚C in a humidified incubator with a gas phase of 5% CO_2. Recombinant human VEGF and bFGF were acquired from PeproTech EC (London, UK). Restriction enzymes *Mlu*I and *Eco*RI were purchased from New England Biolabs (Beverly, MA, USA).

Lentiviral vector production and transduction. Construction of the recombinant lentivirus with alphastatin was performed as previously described (11,12). A four-plasmid-based lentiviral expression system was used in our experiment. Lentiviral shuttle plasmid pWPXL-MOD was a kind gift from the University of California, San Diego. Here, SpNT4 stands for the fusion gene containing the human Neurotrophin-4 signal peptide and pro-region. Briefly, to prepare pseudotyped lentiviral vectors, lent-SpNT4-Al, 20 μg pWPXL/SpNT4-Al, 12 μg pMDLG/pRRE, 10 μg pRSV/REV and 10 μg pMD2.G were co-transfected into 293T cells, which were then cultured in 10-cm dishes with Lipofectamine 2000 (Invitrogen, USA). The lentiviral vector titers were estimated by flow cytometric analyses [fluorescence-activated cell sorting (FACS)] as described previously (13). SHG44 and U87 cells were transduced with Lent-GFP and Lent-SpNT4-Al at the multiplicity of infection of 5-10 in the presence of 6 mg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA) for 72 h. SHG44-GFP (SHG44-Null), SHG44-SpNT4-Al-GFP (SHG44-Al), U87-GFP (U87-Null) and U87-SpNT4-Al-GFP (U87-Al), four types of tranduced cells were harvested.

Determination of the expression of secreted protein alphastatin. Based on the identification of secreted protein alphastatin from HUVECs carrying the alphastatin gene as described previously (11), we assessed whether protein alphastatin was secreted from SHG44 and U87 cells carrying the alphastatin gene in conditioned media using a HUVEC migration assay. HUVEC migration is inhibited when alphastatin is secreted in media. The cell migration assay was adapted from Malinda *et al* (14) and involves the use of a 24-well microchemotaxis chamber (AM; Neuro Probe, Gaithersburg, MD, USA) with 8-μm pore size polycarbonate membranes (Neuro Probe). SHG44, U87, SHG44-GFP, U87-GFP, SHG44-Al and U87 cells were, respectively, cultured in the lower chamber for another 24 h as subconfluent monolayers in DMEM (containing 1% FCS), and HUVEC cell suspension ($1x10^5$ cells/ml) was then added to the upper chambers and incubated at 37˚C for 8 h with VEGF or bFGF alone (10 ng/ml). Migrating cells adhering to the undersurface of the membrane were fixed with 4% paraformaldehyde and stained with hematoxylin and eosin. The migrating cells were then counted using an optical microscope at x20 magnification.

Proliferation assay. The MTT assay was performed as previously described to measure the effects of secreted protein alphastatin on the *in vitro* growth of SHG44 and U87 cells (15). Briefly, SHG44, U87, SHG44-GFP, U87-GFP, SHG44-Al and U87-Al cells were, respectively, seeded into 96-well plates at a density of $3x10^4$ cells/ml in DMEM for 24, 48, 72 and 96 h. At each time point, a quarter volume of MTT solution (2 mg MTT/ml in PBS) was added to each well, and the plates were then incubated for 4 h at 37˚C. The medium was aspirated and the formazan crystals were dissolved in 150 μl of DMSO buffered at pH 10.5. The absorbance was finally read at 590 nm using a Dynex ELISA plate reader (Ashford, Middlesex, UK).

Immunofluorescence. The surface expression and localization pattern of adherens junction proteins was assessed by immunocytochemistry in VEGF- or bFGF-induced endothelial tube formation as previously described (16). HUVECs ($2x10^5$ cells/ml) were incubated for 2 h in 24-well plates coated with 250 μl/well GFR Matrigel, either with or without 20 ng/ml bFGF or VEGF, in conditioned media that were collected from SHG44-Al or U87-Al cells, each of which was cultured until subconfluence in DMEM media for 24 h. The medium was then replaced with DMEM containing 1% FCS for a further 24 h. Cells were then washed and fixed with 4% (w/v) paraformaldehyde at room temperature for 10 min and then permeabilized with 0.5% (v/v) Triton and blocked with 5% (w/v) BSA in PBS for 30 min at room temperature. They were then incubated with a monoclonal anti-VE-cadherin antibody (1:100) overnight at 4˚C. Cells were then washed before incubation with FITC-conjugated goat anti-mouse IgG (1:1,000) for 2 h at 37˚C. Finally, cells were washed again before being overlaid with PBS and analyzed using a fluorescence microscope.

Western blot analysis. HUVECs were treated as described in the Immunofluorescence section. For each phase during tube formation HUVECs were then washed twice with ice-cold PBS and lysed in lysis buffer [50 mM Tris-HCl (pH 7.9), 5 mM EDTA, 0.1% SDS, 10% glycerol, 0.2% Triton X-100, 5 μg/ml aprotinin, 1 mM PMSF and one protease inhibitor cocktail tablet]. Protein concentrations were determined using a BCA kit (Pierce, Rockford, IL, USA). Each protein lysate (30 μg) was separated on SDS-PAGE gels and then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The blots were incubated overnight at 4˚C with primary antibodies to VE-cadherin and β-actin, followed by a 1-h incubation with horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using an enhanced chemiluminescence detection system (Amersham Life Science, Little Chalfont, UK).

Tumor growth inhibition. All animal experiments were approved by the Institutional Animal Care and Use Committee of The Fourth Military Medical University. All experiments were performed on 6-week-old Balb/c mice weighing 20 g (n=13) purchased from Jackson Laboratory (Bar Harbor, ME, USA). Animals were anesthetized with an intraperitoneal injection of 10% chloral hydrate before experiments. Then, $2x10^6$ cells/ml (in 100 μl medium) of viable SHG44 or U87 cells were respectively implanted subcutaneously. Three days after the tumor cells were implanted subcutaneously in the

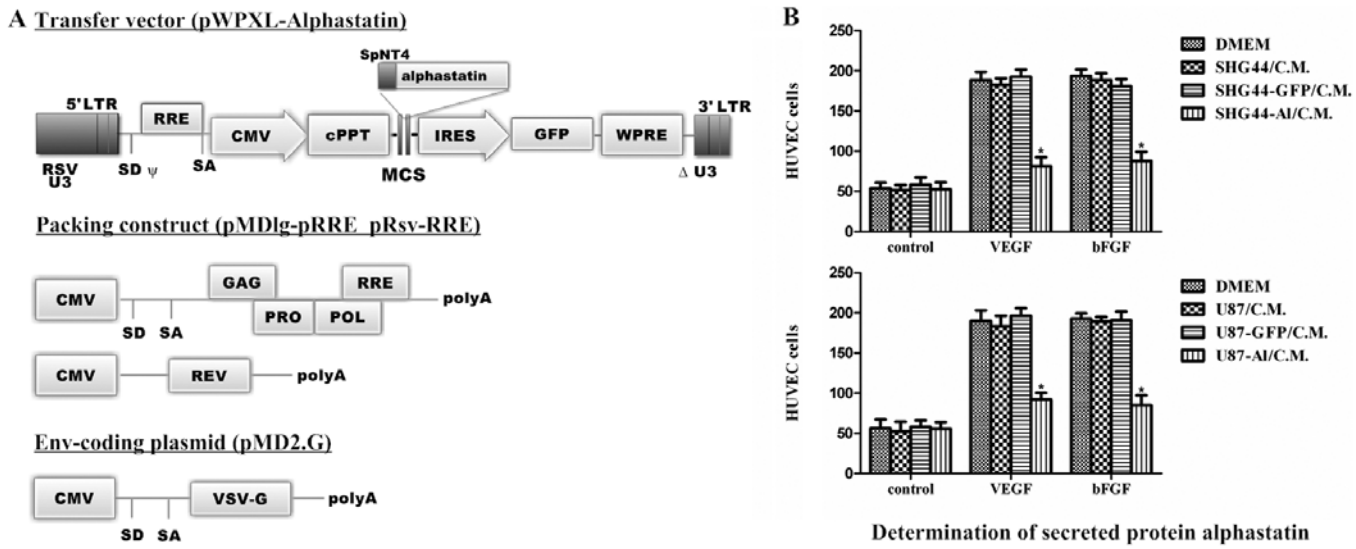

Figure 1. Illustration of the construction and determination of alphastatin expression. (A) Self-inactivating lentiviral vectors: The human neurotrophin-4 signal peptide and pro-region fusion sequences (SpNT4) were cloned into lentiviral transfer vector plasmid pWPXL/GFP, to construct pWPXL-SpNT4-Al. Recombinant lentiviral vectors were produced by cotransfection of the envelope plasmid pMD2.G, packaging plasmid pMDlg-RRE and pRsv-RRE, and transfer vector plasmid. LTR, long terminal repeat; RSV U3, U3 region from Rous sarcoma virus; ψ, packaging signal; SD, splice donor; SA, splice acceptor; RRE, Rev response element; CMV, cytomegalovirus promoter; MCS, multiple cloning site; IRES, internal ribosome entry site; GFP, green fluorescent protein marker gene; ΔU3, self-inactivating deletion in U3 region; polyA, polyadenylation signal; VSV-G, vesicular stomatitis virus G protein envelope. (B) HUVEC migration assay was performed in response to medium or conditioned medium containing or not containing (control) 10 ng/ml VEGF or bFGF, to confirm that the SHG44-Al (upper panel) and U87-Al cells (lower panel) expressed biologically active alphastatin. All data shown are means ± SEM. *P<0.002 compared with the respective VEGF or bFGF group.

mice, recombinant lentNT4/Al (lentivirus-SpNT4-alphastatin) was injected intratumorally twice daily, for two days, with 20 μl 3.4x10^8 TU/ml virus. Tumor volumes were measured daily using calipers (17), along with body weight and the state of well-being.

Immunohistochemical analysis. Mice were sacrificed after 16 days. The tumor tissues were excised, divided in half and fixed overnight in either 10% neutral-buffered formalin or a zinc-based fixative (18), before being embedded in paraffin wax and sectioned. Formalin-fixed tissue sections were subsequently stained with anti-CD31, periodic acid-Schiff (PAS) and hematoxylin to assess microvessel density (MVD). The average vessel count within three selected fields was then scanned at increased magnification (x20), and MVD was expressed as the mean percentage of vessel areas/field from three highly vascularized areas.

Statistical analysis. The data were expressed as the means ± SEM and representative data from one of the three replicate experiments are shown. The differences between the groups were determined using one-way ANOVA followed by the Student's t-test. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Expression of alphastatin in human glioma cells. The recombinant lentivirus vectors for long-term expression of alphastatin were constructed (Fig. 1A). We examined this gene delivery system in SHG44 and U87 human glioma cell lines. Subsequently, SHG44 or U87 cells that were transduced with recombinant lentiviral vectors showed both nuclear and cytoplasmic expression of GFP. Typically at a multiplicity of infection of 5-10, >96% of lentivirally transduced SHG44 cells and >98% of U87 cells are achieved. To confirm that SHG44-Al and U87-Al, but not SHG44-Nul l and U87-Null cells, expressed biologically active alphastatin, we next examined the effect of alphastatin expression in an *in vitro* HUVEC migration assay, in which SHG44-Al and U87-Al cell conditioned media resulted in significant inhibition of endothelial cell migration, whereas conditioned media of SHG44-Null and U87-Null cells did not inhibit endothelial cell migration (Fig. 1B). Based on these data, and combined with previous early-stage results (11), we concluded that the lentiviral vector efficiently transduced alphastatin into the SHG44 and U87 cells, and the SHG44-Al and U87-Al cells secreted active alphastatin.

Effects of alphastatin on the growth of SHG44 and U87 cells. We previously reported that secreted peptide alphastatin inhibited angiogenesis by inhibiting HUVEC migration and tube formation. We further sought to determine whether alphastatin also inhibits the growth ability of human glioma cells following treatment. To evaluate the effects of alphastatin transduction and expression on the growth of SHG44 and U87 cells *in vitro*, the relative growth rates of SHG44-GFP, U87-GFP, SHG44-Al and U87-Al, and parental SHG44 and U87 cells were compared using MTT methods. As shown in Fig. 2, there was no significant difference between the growth rates of SHG44, SHG44-GFP and SHG44-Al cells, suggesting that neither the lentivirus transduction procedure nor the overexpression of either GFP or alphastatin affected the intrinsic rate of cellular proliferation in these cells. Furthermore, in

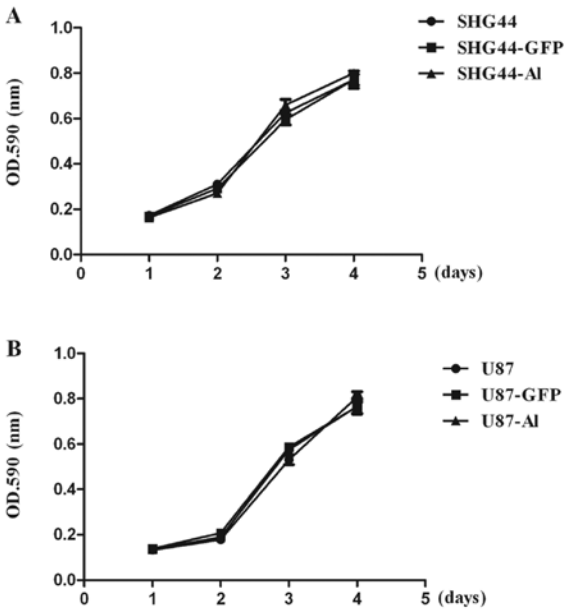

Figure 2. Effect of alphastatin on the growth of (A) SHG44 and (B) U87 cells. Growth rate of SHG44, SHG44-GFP, SHG44-Al U87, U87-GFP and U87 cells was measured by MTT assay. *In vitro* growth characteristics were similar, respectively, in A and B.

contrast, these data confirmed that secreted protein alphastatin did not effect the growth of SHG44 and U87 cells.

Effects of alphastatin on VE-cadherin. Since VE-cadherin is crucial for controlling the state of adherens junctions, which in turn regulate endothelial cell-cell adhesion, cell motility, morphogenesis and intracellular signaling pathways, this molecule has many clinical implications. To gain insight into one possible molecular mechanism involved in the inhibition of angiogenesis by alphastatin, we sought to determine whether alphastatin influences the expression and distribution of VE-cadherin during HUVEC tube formation. Immunofluorescence analysis showed that VE-cadherin was localized along areas of cell-cell contact. However, 1-2 h after stimulation by VEGF or bFGF, the VE-cadherin fluorescence was greatly reduced, and even disappeared from most cell-cell contact areas. Whereas, when cells were treated with SHG44-Al or U87-Al conditioned media prior to stimulation with VEGF or bFGF, VE-cadherin maintained localization along areas of cell-cell contact (Fig. 3A). Furthermore, we measured the total amount of VE-cadherin during HUVEC tube formation using western blot analysis and found no significant changes in the levels of total VE-cadherin protein (Fig. 3B). These findings suggest that alphastatin inhibits the decrease in VE-cadherin induced by VEGF or bFGF on the surface of HUVECs without any changes in the total protein of VE-cadherin.

Effects of alphastatin on tumor growth. We examined the inhibitory effect of secreted protein alphastatin on tumor growth *in vivo*. SHG44 or U87 cells were subcutaneously implanted alone in mice. In all mice, SHG44 tumors grew to a volume between 62 and 1,644 mm^3, and U87 tumors grew to a volume between 63 and 1,904 mm^3 after 16 days of implantation. However, SHG44 tumors treated with lentNT4/Al showed a significantly reduced growth rate, reaching a final tumor volume of only 734±79 mm^3 after 16 days ($P<0.018$; Fig. 4A). The growth rate of U87 tumors was also significantly reduced, reaching a final volume of only 827±92 mm^3 ($P<0.022$; Fig. 4B). Similar results were obtained in two repeated experiments. Alphastatin was found to be well tolerated by the animals, and had no significant effect on body weight or general well-being (animals were not lethargic, and intermittent hunching, tremors, or disturbed breathing patterns were not observed). These data suggest that secreted protein alphastatin effectively suppresses tumor growth in an established mouse tumor model.

Effects of alphastatin on tumor histology. Histologic analysis revealed a difference in MVD between the two implanted xenograft tumor groups. MVD was determined by the examination of CD31-stained sections at those sites with the highest number of capillaries and small venules. The positive expression of CD31 was visualized as brown-yellow or brown granules in the cytoplasm of the vascular endothelial cells. In the SHG44 tumors injected with the recombinant lentNT4/A1, the MVD was significantly reduced (lentNT4/Al + SHG44 vs. SHG44, 10.33±1.453 vs. 25.33±2.028; $P<0.039$) (Fig. 4A). In U87 tumors injected with the recombinant lentNT4/A1, the MVD was also significantly reduced (lentNT4/Al + U87 vs. U87, 11.33±1.764 vs. 27.64±1.475; $P<0.001$) (Fig. 4B). The results suggest that alphastatin significantly inhibits tumor angiogenesis, and that secreted protein alphastatin effectively suppresses tumor growth in an established tumor model, as a result of antiangiogenetic effects.

Discussion

Alphastatin is an endogenous angiogenesis inhibitor with potent antiangiogenic properties which inhibits both the migration and tube formation of human endothelial cells induced by VEGF or bFGF, and in tumor models by inhibiting tumor growth through suppressing angiogenesis. Using lentivirus-mediated gene transfer, we previously found that lentNT4/Al-infected HUVECs were able to stably secrete alphastatin, which exhibited a potent inhibitory effect on HUVEC migration and differentiation. We also explored the inhibitory effects of secreted protein alphastatin on tumorigenesis in glioma, in which secreted protein alphastatin significantly suppressed tumorigenesis, since the influence of alphastatin on the growth of glioma cells and the effects of alphastatin therapy on established tumors remain unclear. Here, we demonstrated in SHG44-Al and U87-Al cells and an established treatment model for human glioma that lentivirus-mediated gene transfer of alphastatin is a potential therapeutic strategy against glioma and, alphastatin inhibits tumor growth by blocking VE-cadherin turnover.

Lentiviral vectors offer the advantage that they can integrate their cDNA into both dividing and nondividing cells (19). In addition, VSV-G pseudotyped lentiviral vectors, which can bind to cell surface phospholipids (20), have a potential advantage in achieving efficient gene delivery to endothelial cells. Furthermore, a third-generation SIN lentiviral vector, in which the U3 region of the 3' LTR (including the TATA box) was deleted, abolishing any LTR promoter activity (21), enhanced

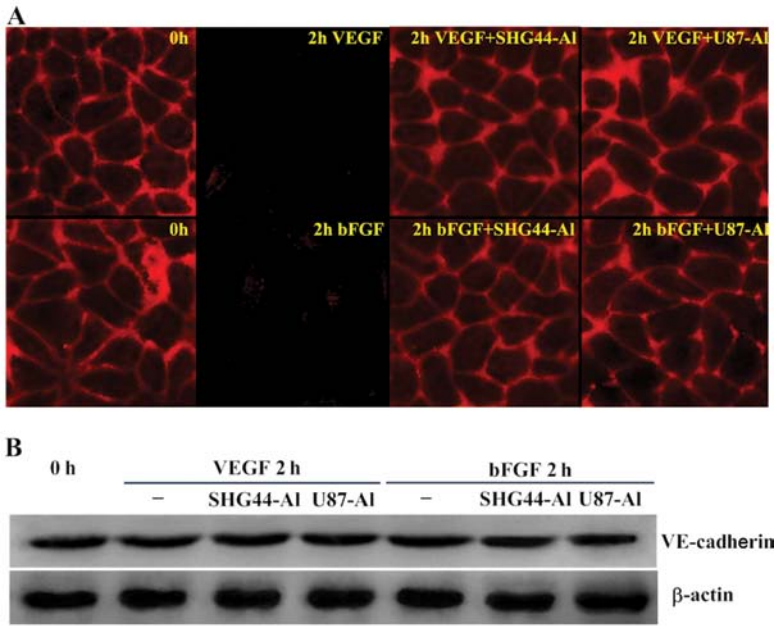

Figure 3. Effect of alphastatin on VE-cadherin. Adherence junction molecule VE-cadherin was detected during endothelial cell capillary tube formation induced by 10 ng/ml VEGF or bFGF at 0 and 2 h, and treated with or without alphastatin conditioned medium. (A) Immunolocalization of VE-cadherin by immunofluorescence microscopy; magnification, x400. (B) VE-cadherin expression levels were analyzed by western blotting. β-actin was used as a loading control.

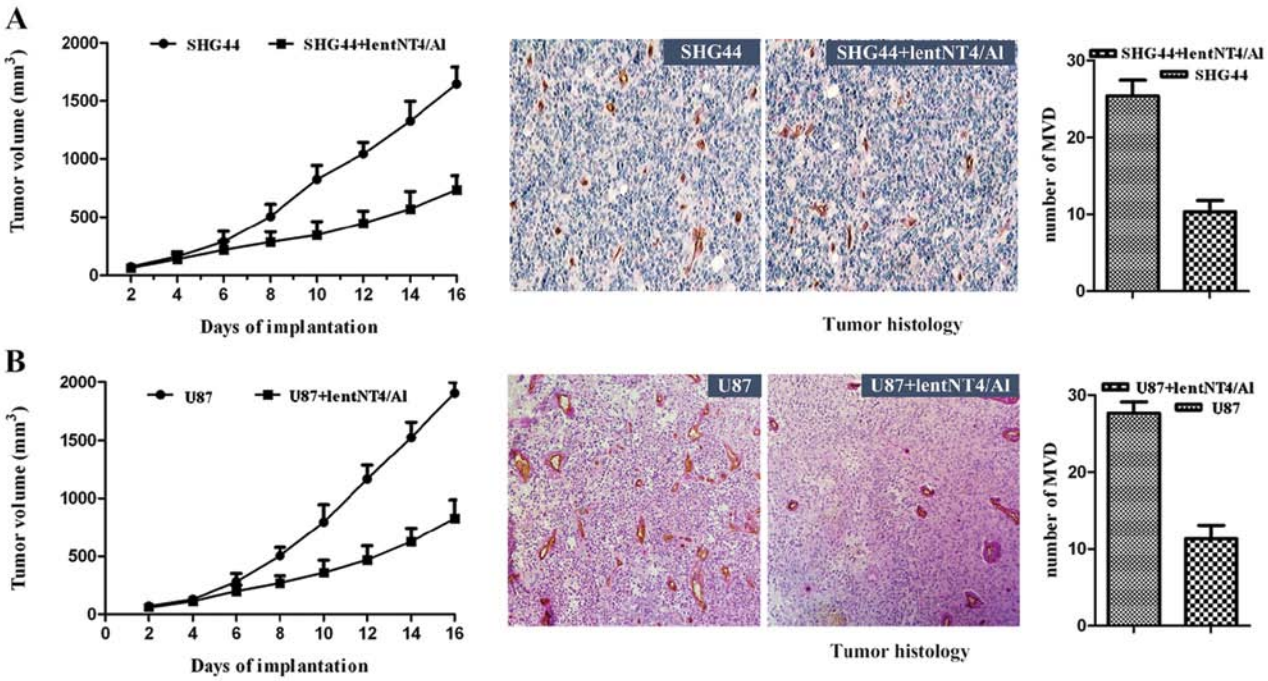

Figure 4. Effects of alphastatin on tumor growth and tumor histology. (A) The line graph shows the changes in SHG44 tumor volume over 16 days. Data are expressed as means ± SEM. P<0.018, SHG44 + lentNT4/Al vs. SHG44. The MVD values are expressed as means ± SEM. P<0.039; SHG44 + lentNT4/Al vs. SHG44 (original magnification, x20). (B). The line graph shows the changes in U87 tumor volume over 16 days. Data are expressed as means ± SEM. P<0.022; U87+ lentNT4/Al vs. U87. The MVD values are expressed as means ± SEM. P<0.001; U87 + lentNT4/Al vs. U87 (original magnification, x20).

the security level of transgene expression. Accordingly, the NT4 signal peptide and pro-region sequence were fused in-frame to the 5' ends of each coding sequence to ensure exogenous protein expressing in a secretory manner (22-26), and the lentNT4/Al gene delivery system was constructed. Moreover, since previous results showed that secreted protein alphastatin had significantly attained a saturated concentration of antiangiogenesis in respective conditioned medium (11), there was no concern with variation of concentration of secreted protein in this study.

Since secreted protein alphastatin is sustainably expressed in HUVEC-Al cells, and *in vitro* and in glioma models significantly inhibit angiogenesis and gliomagenesis, we aimed to

ascertain whether alphastatin also remains stably expressed in SHG44-Al and U87-Al cells. We investigated its anti-glioma activity involving its efficacy in the treatment of glioma, and the analysis of the treatment mechanism. In view of the successful construction of the lentiviral vector containing the NT4-alphastatin fusion gene and sustainable expression of alphastatin in HUVEC-Al cells, in this study, we constructed SHG-44-Al and U87-Al cells, and by morphological observation confirmed whether alphastatin was secreted in these cells. We showed that, in an endothelial migration assay, SHG-44-Al and U87-Al conditioned media had a similar inhibitory effect on endothelial cell migration, which suggested that alphastatin was successfully secreted in SHG-44-Al and U87-Al cells. Notably, although injection of the recombinant lentNT4/Al significantly inhibited tumor growth, our data also showed that secreted protein alphastatin did not affect the proliferative ability and migration of glioma cells *in vitro*. This may be due to the fact that alphastatin acts only on endothelial cell angiogenesis, but not on glioma cells. Taken together, our data suggest that secreted protein alphastatin is stably expressed in SHG44-Al and U87-Al cells, leading to growth suppression of glioma by inhibition of endothelial cell migration and tube formation.

VE-cadherin is a major component of endothelial adherens junctions necessary for blood vessel integrity and endothelial cell survival (27,28). VE-cadherin has emerged as an adhesion molecule that plays fundamental roles in microvascular permeability and in morphogenic and proliferative events associated with angiogenesis (29-31). Since blocking angiogenesis is a common strategy with which to inhibit tumor growth, several approaches to inhibiting VE-cadherin function have been suggested as possible antitumor therapies (32-35). Several studies have demonstrated that VE-cadherin turnover in endothelial cells suppresses angiogenesis (16,36-39), and is closely related to the MAPK pathways (40,41). Our finding that alphastatin inhibits VE-cadherin downregulation induced by VEGF or bFGF on the surface membrane of HUVECs without any changes in total protein of VE-cadherin suggests that VEGF or bFGF induces VE-cadherin redistribution in endothelial cells, which is inhibited by alphastatin. Our previous data also indicate that secreted protein alphastatin inhibits VEGF- or bFGF-induced angiogenesis by suppressing the JNK and ERK kinase activation pathways in HUVECs. We previously confirmed that the antiangiogenetic activity of alphastatin most likely operates at a post-receptor locus common to the VEGF and bFGF signaling pathways (9). Thus, our data indicate that the antiangiogenesis mechanism of alphastatin, by blocking VEGF and bFGF post-receptor signaling pathways, suppresses JNK and ERK kinase activation, which lead to VE-cadherin turnover on the endothelial cell membrane, and then, inhibits endothelial cell migration and differentiation. Finally, alphastatin not only inhibits new vessel growth, but more important it significantly causes regression of existing tumor vessels.

In conclusion, in our study of antiangiogenesis of alphastatin, we describe the anti-glioma therapeutic strategy involving lentivirus-mediated gene transfer of alphastatin and its role in the inhibition of tumorigenesis. We have demonstrated that *in vivo*, a subcutaneous injection of recombined lentiNT4-Al resulted in the significantly growth inhibition of glioma in autocrine manner (HUVEC-Al cells) or in paracrine manner (in SHG-44-Al and U87-Al cells), and *in vitro*, offering a molecular explanation for the mechanism of the effect of alphastatin against glioma involving VE-cadherin. This study is the first report on an anti-glioma therapeutic strategy involving lentivirus-mediated gene transfer of alphastatin.

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