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Valproic Acid Inhibits Angiogenesis In Vitro and Glioma Angiogenesis In Vivo in the Brain

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Abstract

Antiangiogenic strategy is promising for malignant glioma. Histone deacetylase inhibitors (HDACIs) are unique anticancer agents that exhibit antiangiogenic effects. The in vitro and in vivo antiangiogenic effects of HDACIs, valproic acid (VPA), were investigated in malignant glioma in the brain. In vitro, VPA preferentially inhibited endothelial cell proliferation compared to glioma cell proliferation at the optimum concentration in a dose-dependent manner. VPA reduced vascular endothelial growth factor (VEGF) secretion of glioma cells in a dose-dependent manner under both normoxic and hypoxic conditions. VPA was also found to inhibit tube formation in the angiogenesis assay. In vivo, treatment with VPA combined with irinotecan reduced the number of vessels expressing factor VIII in the brain tumor model. VPA inhibits glioma angiogenesis by direct (inhibition of endothelial cell proliferation and tube formation) and indirect (decreased secretion of VEGF by glioma cells) mechanisms. These data suggest a potential role for VPA as an adjuvant therapy for patients with malignant glioma.

Key words: histone deacetylase inhibitor, valproic acid, glioma, angiogenesis, vascular endothelial growth factor

Introduction

Malignant glioma remains a uniformly fatal disease, despite advances in surgical techniques and drugs. Malignant glioma tends to be highly vascularized and to contain hypoxic regions. Therefore, an antiangiogenic strategy is a promising approach for the treatment of malignant glioma.[11,27] In recent years, treatment with vascular endothelial growth factor (VEGF) antibody has demonstrated an effect to extend the duration to recurrence.[26] However, the effect of treatment remains inadequate, and establishment of a highly effective regimen is needed.

Histone deacetylase inhibitors (HDACIs) are a promising new class of anticancer agents. Acetylation and deacetylation of histone plays an important role in epigenetic regulation of gene expression of cancers. HDACIs inhibit the histone deacetylases that induce nucleosomal histone deacetylation, eukaryotic chromatin condensation, and gene expression silencing.[30] The effects of HDACIs are not limited to histone deacetylation inhibitors. They also act as members of protein complexes to recruit transcription factors of tumor suppressors and affect the acetylation status of specific cell cycle regulatory proteins.[2] HDACIs have been proven to affect proliferation, differentiation, apoptosis, and anti-angiogenesis in solid tumors including gliomas.[10,18,21] Several studies have shown that the HDACIs exhibit direct inhibitory effects on endothelial cell proliferation and angiogenesis in vitro and in vivo.[9,17] Other reports have also indicated that HDACIs inhibit new blood vessel formation by down-regulating angiogenesis-related gene expression in endothelial and tumor cells.[20]

Valproic acid (VPA) has helped to identify the underlying mechanism of HDACIs.[19] VPA is a conventional drug with proven efficacy to modify apoptosis, growth arrest, and cell differentiation in cancer cells through inhibition of histone deacetylase.[5,15] VPA also inhibits tumor angiogenesis in vivo in xenograft models of medulloblastoma, neuroblastoma, prostatic cancer, and colon cancer.[8,22,29,31] However, the anti-angiogenic and anti-tumor actions of VPA have not been fully investigated against

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malignant gliomas.

This study demonstrated the anti-angiogenic effects of VPA on malignant glioma both in vitro and in vivo in the brain.

Materials and Methods

The human glioma cell lines, U87-MG, U251, and A172, and the rat glioma cell line, C6, were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The human glioma cell line, U343, was a gift from Dr. Mark Penfold (ChemoCentryx, Mt. View, California, USA). The immortalized human umbilical vein endothelial cell line, TE-1, was a gift from Dr. Yoji Mitsui (Tokushima Bunri University, Tokushima). U87-MG, U251, A172, U343, C6, and TE-1 were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 5% penicillin-streptomycin solution (Sigma, St. Louis, Missouri, USA). At each passage, cells were harvested as single-cell suspensions using trypsin/ethylenediaminetetraacetic acid. U87-MG, U251, A172, and C6 were cultured in a Falcon® flask (Becton Dickinson, Franklin, New Jersey, USA). TE-1 was cultured in a collagen type I coated flask (Iwaki, Tokyo). Cells were incubated in both normoxic and hypoxic conditions. Normoxia was set as 21% O_2 and 5% CO_2, hypoxia 1.0% O_2 and 5% CO_2 using a multi gas incubator (APM-30D; Astec Co., Ltd., Fukuoka). VPA was obtained from Kyowa Pharmaceutical Industry Co., Ltd. (Osaka) and irinotecan (CPT11) from Daiichi-Sankyo Co., Ltd. (Tokyo).

To investigate the effect of VPA on cell growth, the WST-8 assay kit (Kishida Chemical Co., Ltd., Osaka) was used. Cells (5×10^3 cells/well in a 96 well plate) were incubated overnight. The medium was then exchanged for new medium with various concentrations of VPA (0, 400, 800, 1000, 2000, 3000, 4000, 6000 μM). After 48-hour incubation, the WST-8 reagents were added. After 90-minute incubation, the absorbance at 450 nm was measured with a Model 550 microplate reader (Bio-Rad Laboratories, Hercules, California, USA). The following formula was used for calculations:

% Control =
(each absorbance − absorbance of the blank well)/
(absorbance of 0 μM well)

U87-MG, U251, and U343 cells were seeded at a density of 5×10^4 cell/well and incubated. Cells were treated with medium containing with 0, 500, 2000, and 5000 μM VPA. Conditioned medium was collected after incubation for 24, 48, 72, and 96 hours under normoxic conditions. VEGF concentrations were quantified by Quantikine® human VEGF immunoassay kit (R&D Systems, Minneapolis, Minnesota, USA).

Evaluation of angiogenesis was performed by assessing the occurrence of microvessel structures (tube formation) from human umbilical vein endothelial cells (HUVECs) using an angiogenesis kit (Kurabo Industries Ltd., Osaka). Evaluation of angiogenesis, using an angiogenesis kit, was performed in vitro by assessing the occurrence of microvessel structures from HUVECs pre-seeded in a 24-well plate and co-cultured with fibroblasts cells. When HUVECs reached the early stage of neovascular formation, the medium was changed to an angiogenesis medium containing 10 ng/ml VEGF-A for control wells. At the same time, the medium with various concentrations of VPA (0, 500, 1000, 2000, 3000 μM) was changed on days 4 and 7 of culture. Furthermore, suramin at 50 μM, which is known to be an angiosuppressive drug, was assayed as a negative control. On day 10 of incubation, cells were fixed in 70% ethanol and washed with phosphate buffered saline (PBS) containing 1% bovine serum albumin. Cells were incubated for 1 hour with anti-human CD31 antibody (dilution of 1:4000) and goat anti-mouse immunoglobulin G (IgG) (dilution of 1:500). Cells were washed with distilled water and 5-bromo-4-chloro–3-indoryl phosphate/nitroblue tetrazolium solution was added to stain the vascular wall. Five fields per well were photographed under a microscope, and vessel lengths were assessed using WinROOF analyzing software (Mitani Corp., Fukui).

Five-week-old female Wister rats were inoculated with 1×10^6 C6 cells into the right frontal lobe. Two experiments were planned. The first experiment was designed to evaluate the anti-proliferative effect of only VPA in 15 rats. Rats were treated with VPA 0, 200, and 400 mg/kg once a day, intraperitoneally ($n = 5$ each) for 28 days from 1 week before the tumor injection. In this experiment, body weights of control rats, VPA low dose (200 mg/kg) rats, and VPA high dose (400 mg/kg) rats at 24 days after implantation were 197.0 ± 40.4, 199.3 ± 24.8, and 153.0 ± 9.8 g, respectively. Rat body weight was significantly lower in the VPA high dose group compared to the VPA low dose group and the control group, suggesting systemic toxicity with high dose of VPA. We chose low dose VPA (200 mg/kg) for further experiments. Because our previous experiment demonstrated that metronomic treatment with CPT-11 resulted in glioma inhibition with dramatic angiosuppressive action,[23] we designed the next experiment in which VPA was combined with metronomic CPT-11 administration to evaluate the synergistic or additive effect of angiosuppression for glio-

ma growth. Twenty-one rats were divided into three groups (7 rats each), control, only VPA (200 mg/kg), and VPA concomitant with metronomic CPT-11 treatment (1 mg/kg, intraperitoneally once a day for 24 days after the tumor injection). All rats were sacrificed at 25 days after the tumor injection when many of the control rats were in a moribund state. Tumor volumes were measured using the following formula: tumor volume = (length × width^2)/2. The tumor bearing brain was removed, cut in coronal sections, fixed in 10% formalin, and then embedded in paraffin. Animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experimental Committee of the University of Tsukuba and in accordance with the University's Regulation for Animal Experiments.

The Dako LSAB kit for mouse and rabbit primary antibody (Dako, Glostrup, Denmark) was used. The tissue sections (5 μm thickness) were deparaffinized and incubated with 10% normal goat serum in PBS for 20 minutes. The sections were then incubated with monoclonal anti-mouse factor VIII antibody (Sigma) and polyclonal anti-rabbit VEGF antibody (A-20; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) in PBS for 60 minutes at room temperature. Chromatographically purified mouse and rabbit IgG (Dako) at the same IgG concentration was used as a negative control. Sections were incubated with biotin-conjugated goat anti-mouse immunoglobulin for 10 minutes, followed by washing in PBS for 10 minutes. The sections were then incubated with peroxidase-conjugated streptavidin solution for 5 minutes, followed by washing in PBS for 5 minutes. Sections were then stained with freshly prepared aminoethyl carbazole solution for 10 minutes, followed by washing for 5 minutes in tap water. The sections were finally counterstained with hematoxylin and mounted with aqueous mounting media. Five fields per tumor were photographed under a microscope, and the cells that became positive for factor VIII were measured automatically using WinROOF analyzing software.

Statistical analysis was performed using the Student t-test (paired and unpaired) for continuous variables where we observed mean differences between and within groups. To determine the significant differences between the groups including intermittent or categorical variables, the Mann-Whitney (unpaired) or Wilcoxon (paired) tests were used. Statistical significance was considered at $p \leq 0.05$.

Results

VPA inhibited endothelial cell (TE-1) proliferation more than glioma cell (U87-MG, U251, A172, C6) proliferation at optimum concentrations (800–4000 μM), which match the therapeutic concentrations achieved during routine treatment (Fig. 1). The calculated half maximal inhibitory concentration for VPA were 6034, 8133, 122000, 4254, and 2030 μM for U87-MG, U251, A172, C6 glioma cells, and TE-1 endothelial cells, respectively.

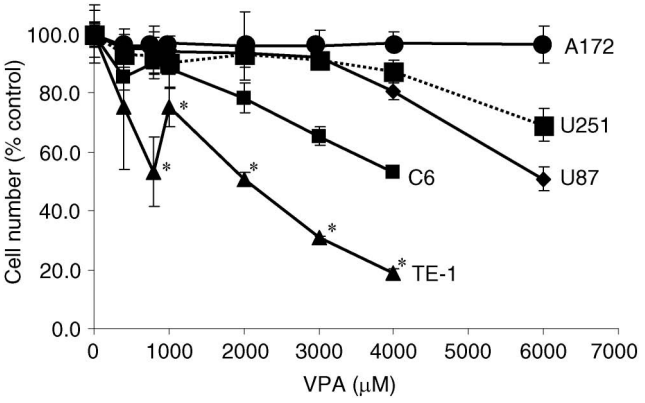

Fig. 1 Inhibition of cell proliferation with valproic acid (VPA) for glioma cells (U87, A172, U251, C6) and endothelial cells (TE-1). The inhibition of endothelial cell proliferation was prominent compared to glioma cell proliferation, at optimum concentrations without significant cell toxicity. *$p < 0.05$, Mann-Whitney test.

To evaluate the effect of VPA on VEGF secretion, VEGF concentrations were measured in conditioned media of U87, U251, and U343 glioma cells under normoxic conditions. VPA significantly reduced VEGF secretion in a dose-dependent manner at each time period with U87 cells (Fig. 2A, $p < 0.05$). VPA significantly reduced VEGF secretion in doses of 2000 and 5000 μM at 72 and 96 hours incubation with U251 cells (Fig. 2B, $p < 0.05$). By contrast, U343 secreted little VEGF in the conditioned medium. Therefore, the VPA inhibitory effect on VEGF secretion was not significant with each dose at each time with U343 cells (Fig. 2C).

VPA inhibited HUVEC tube formation in a dose-dependent manner at concentrations ranging from 500 to 2000 μM (Fig. 3A–E). The inhibition was statistically significant under all conditions (Fig. 3F).

A brain tumor model established from C6 cell lines was used to investigate the antitumor effects of VPA and CPT-11. All rats tolerated the treatment well and had C6 glioma in the brain. Tumor growth was significantly inhibited with single-agent VPA therapy, and enhanced effects were seen using combination therapy with CPT-11 (Fig. 4).

Immunolocalization of VEGF in the brain tumor sections demonstrated that cytoplasmic localization

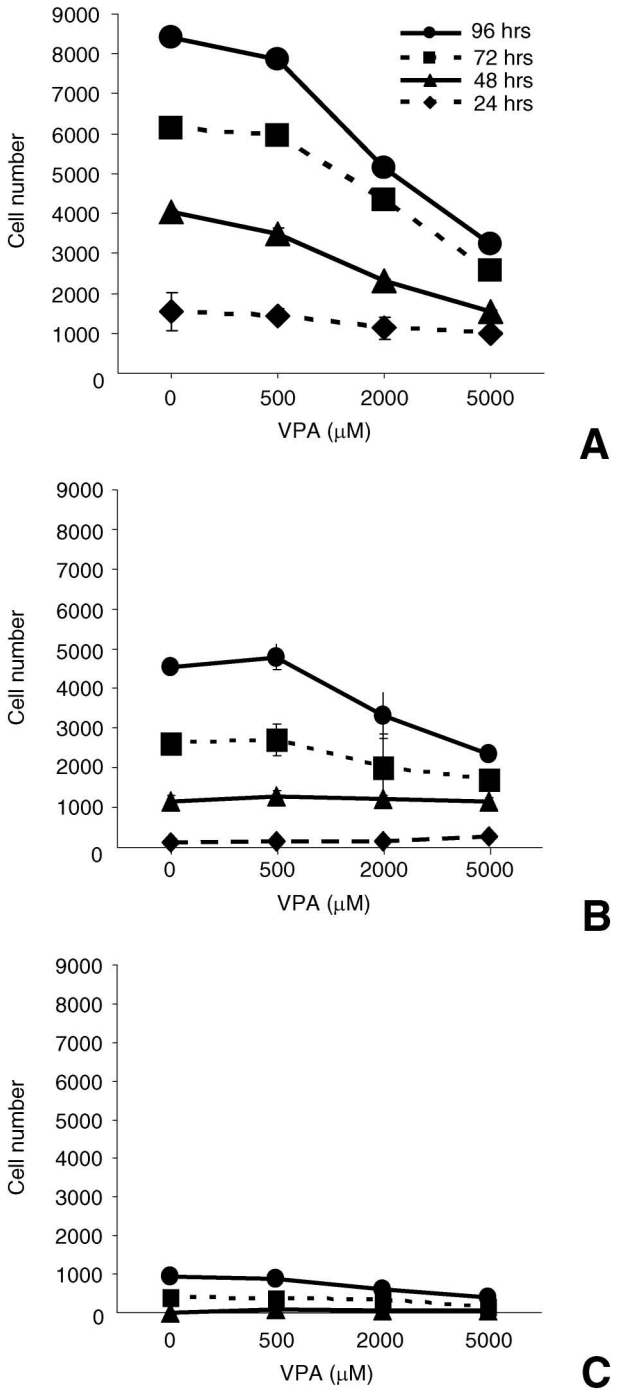

Fig. 2 Inhibition of vascular endothelial growth factor (VEGF) secretion into the conditioned media with valproic acid (VPA) for glioma cells (U87, U251, U343). A: VPA reduced significantly VEGF secretion in a dose-dependent manner at each time period with U87 cells. B: VPA significantly reduced VEGF secretion in doses of 2000 and 5000 μM at 72 and 96 hours incubation with U251 cells. C: U343 secreted little VEGF in the conditioned medium. Therefore, the VPA inhibitory effect on VEGF secretion was not significant with each dose at each time with U343 cells.

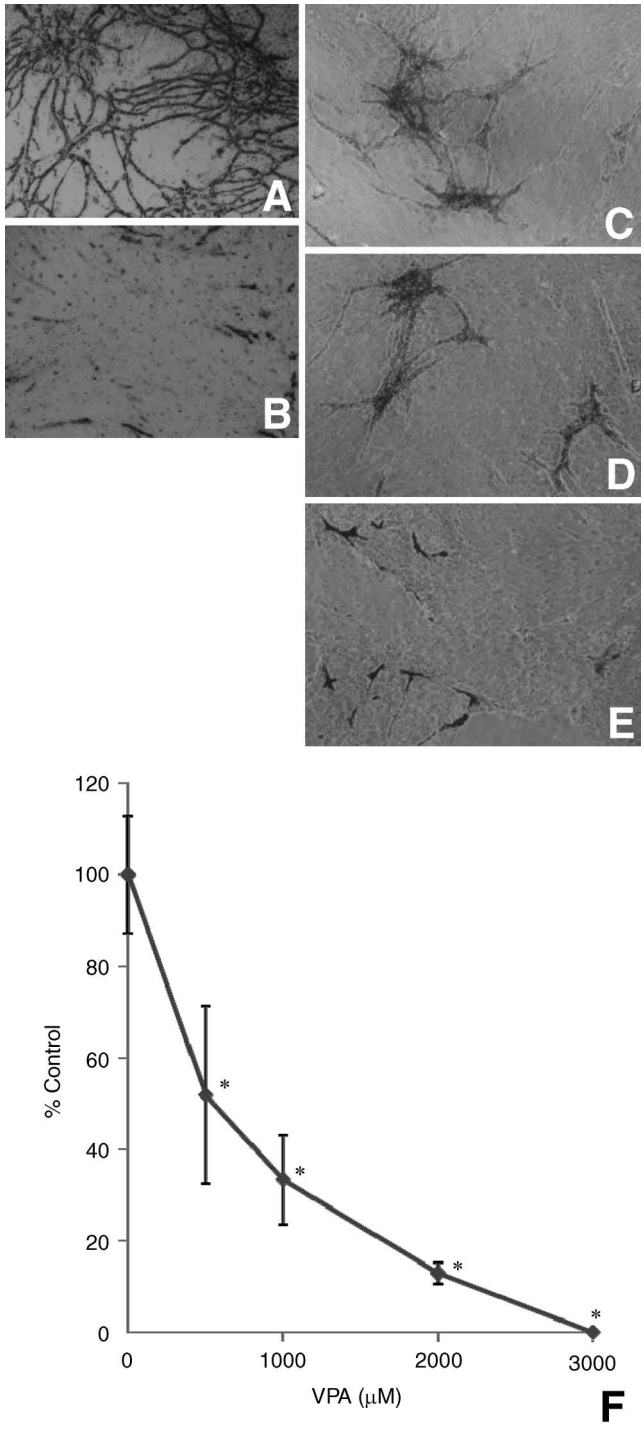

Fig. 3 Inhibition of tube formation assay with valproic acid (VPA). A–E: Tube formation from human umbilical vein endothelial cells was inhibited by VPA in a dose-dependent manner at concentrations of 500 (C), 1000 (D), and 2000 μM (E). A: VPA 0 μm (control), B: suramin 50 μm (negative control). 5-Bromo-4-chloro-3-indoryl phosphate/nitroblue tetrazolium stain, original magnification $\times$50. F: Due to assessments using software, these inhibitions were statistically significant between all conditions. *$p < 0.05$, Mann-Whitney test.

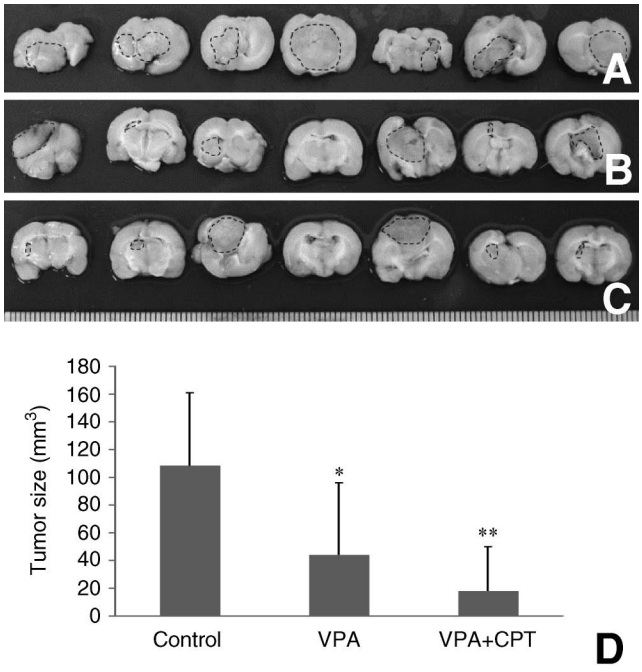

Fig. 4 Antitumor effects of valproic acid (VPA) and CPT-11 in vivo in the brain tumor model. A–C: Macroscopic tumor growth in control (A), VPA (B), and VPA + CPT-11 groups (C). Tumor margin is outlined with a *dotted line*. D: Tumor size at sacrifice is demonstrated in each group (mean ± standard deviation). Tumor growth was significantly inhibited with VPA single-agent therapy ($^{*}p < 0.05$), and the inhibitory effect was enhanced with combination therapy with metronomic CPT-11 ($^{**}p < 0.01$).

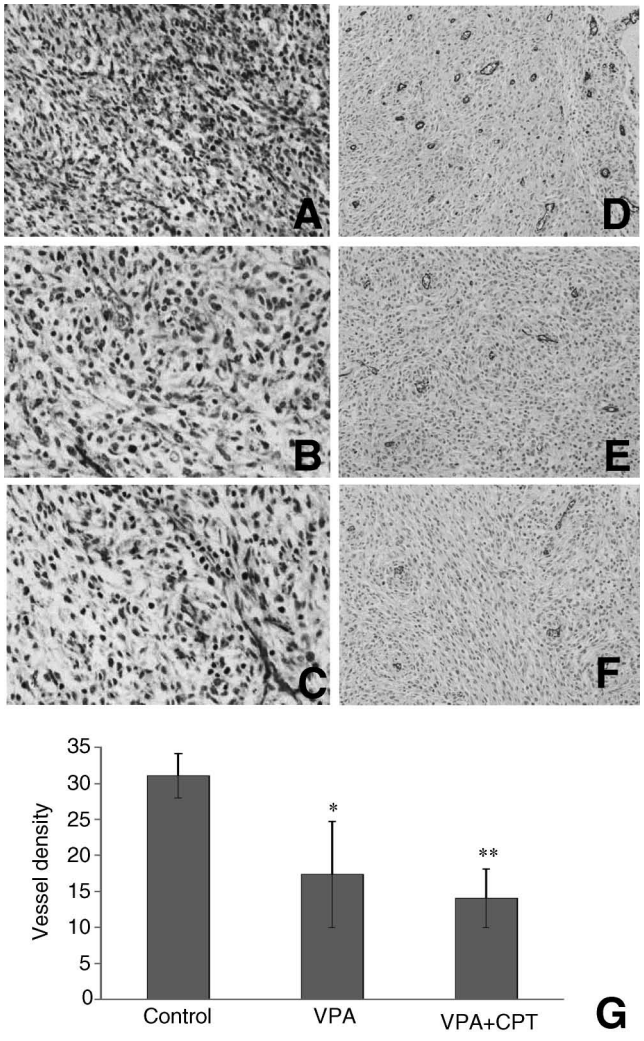

Fig. 5 Anti-angiogenic effect of valproic acid (VPA) and CPT-11 in vivo in the brain tumor model. A–C: Tumor cell expression of vascular endothelial growth factor is prominent in control (A), but reduced in VPA (B) and VPA + CPT-11 (C). Counterstained with hematoxylin, original magnification ×100. D–F: Vessel densities (the number of factor VIII-positive vessels) in the tumors in the control group (D), the VPA group (E), and combination of CPT-11 and VPA treatment group (F). Vessel density is reduced with VPA and VPA + CPT-11 treatment compared to the control. Counterstained with hematoxylin, original magnification ×100. G: These differences are statistically significant due to assessments using software ($^{*}p < 0.01$, $^{**}p < 0.001$ compared to control).

of VEGF in tumor cells was predominantly inhibited after the treatment with VPA and VPA + CPT-11 (Fig. 5A–C). Vessel densities (i.e., the number of factor VIII-positive vessels) were significantly attenuated with VPA and VPA + CPT-11 treatment (Fig. 5D–G).

Discussion

This study evaluated the effects of VPA on angiogenesis in vitro and on glioma angiogenesis in vivo. As expected, VPA inhibited human endothelial cell growth and human endothelial tube formation, and reduced VEGF secretion of glioma cells into the conditioned medium in vitro. Treatment with only VPA reduced factor VIII-positive vessel densities and tumor growth in vivo in the brain tumor model. Moreover, these anti-angiogenic and anti-tumor effects were enhanced with the treatment with VPA combined with CPT-11. These results showed two important findings. First, VPA appeared to inhibit glioma angiogenesis by two mechanisms: ''direct and indirect.'' The indirect mechanism occurred through inhibition of VEGF protein secretion by glioma cells in vitro (Fig. 2A, B) and in vivo (Fig. 5A–C). Because secreted VEGF is a major angiogenic factor in glioma, VPA inhibition of VEGF secretion in vitro and VEGF expression in vivo in the

brain are important actions of indirect angiosuppression of VPA.[1,4] The direct mechanism represents a direct effect on endothelial cells in terms of endothelial cell proliferation and tube formation. Second, combination of other anti-angiogenic agents, such as metronomic CPT-11 treatment, enhanced the anti-angiogenic and anti-tumor effects of VPA. Therefore, VPA is a candidate angiosuppressive agent for malignant gliomas.

VPA has been well established as one of the HDACIs. HDACIs can inhibit angiogenesis of various tumors through many mechanisms. HDACIs affect numerous pro- and anti-angiogenic genes.[16] HDACIs suppress the over-expression of hypoxia-inducible factor-1alpha (HIF-1α) by both direct and indirect mechanisms. The direct mechanisms act through the degradation and loss of HIF-1α transcriptional activity, and proteasomal degradation independent of von Hippel-Lindau protein (pVHL).[13] The indirect mechanisms act through the suppression of p53 and pVHL,[12] acetylation of p300 causing its disassociation and degradation of HIF-1α,[7] and hyperacetylation of Hsp90, which results in the accumulation of immature HIF-1α/70-kDa heat shock protein complexes.[14] Many studies have been performed to elucidate other anti-angiogenic genes that are altered by HDACIs. The pro- and anti-angiogenic genes altered by HDACIs are as follows: p53, pVHL, VEGF, activin A, basic fibroblast growth factor (bFGF), thrombospondin1, endothelial nitric-oxide synthase (eNOS), angiopoietin-1 and -2, and tunica intima endothelial kinase 2.[6] Regarding the angiosuppressive action of VPA, VPA inhibits the expression of VEGF and FGF in colon carcinoma.[31] Previous reports also showed that VPA inhibited angiogenesis; endothelial cell proliferation, endothelial cell tube formation, and decrease in eNOS expression.[9,17] One study indicated that VPA treatment resulted in profound decreases in the proliferation of a prostate cancer cell line, not only by increasing histone H3 acetylation and up-regulating p21CIP1/WAF1 expression, but also by down-regulating VEGF expression.[8] VPA at high dose also inhibited the proliferation of two glioma cell lines (U251 and U87, Fig. 1), suggesting the role of histone deacetylase on glioma cell proliferation. VPA is also important in HIF-1-induced tumor angiogenesis through the inhibition of HDAC1 and HDAC3, which is considered as a positive regulator of HIF-1α.[13] Our present study proved that VPA inhibited glioma angiogenesis by mechanisms similar to those mentioned above.

These positive results of VPA raise the question of whether the usage of VPA as an anti-epileptic drug in brain tumor patients results in prolonged survival compared to patients without VPA usage. VPA has been used clinically in patients for a long time. Could these patients be influenced by VPA? Past studies have answered a part of this question. One researcher reported that there was no remarkable difference in survival between the patients with and without VPA.[25] However, this study was not a randomized control study and has the problem that the ratio of high-grade glioma was not similar between the two groups. Another report demonstrated that VPA was well tolerated in heavily pretreated pediatric patients with high-grade glioma with moderate tumor efficacy.[28] Furthermore, most studies in the past which examined the clinical effect of VPA did not include therapy with antiangiogenesis drugs, such as bevacizumab. Further research will be needed to investigate the combination therapy of VPA with other antiangiogenesis agents. Bevacizumab had been successful in prolonging survival in glioma patients, but this effect was transient and resistance to bevacizumab is the most important problem to be resolved.[24] Because one of the mechanisms of the resistance to bevacizumab treatment is activation of another angiogenic pathway,[3] VPA might be suitable for use in combination with bevacizumab. In this study, we demonstrated the effect of combination of VPA with another angiosuppressive agent, CPT-11, in preventing glioma angiogenesis. Therefore, stronger HDACIs combined with other angiogenesis inhibitors may be promising in the treatment of patients with malignant gliomas in the future.

VPA inhibits angiogenesis by mechanisms involving a decrease in VEGF expression and inhibition of tube formation. VPA could be useful as an adjuvant treatment for malignant glioma through its anti-angiogenic action.

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