Effects of all-trans retinoic acid on VEGF and HIF-1α expression in glioma cells under normoxia and hypoxia and its anti-angiogenic effect in an intracerebral glioma model

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Abstract. All-trans retinoic acid (ATRA) is one of the most potent inducers of differentiation and is capable of inducing differentiation and apoptosis in glioma cells. However, the effect of ATRA on glioma angiogenesis is yet to be elucidated. The present study investigated the effects of ATRA on the expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) in various glioma cell lines under normoxia and hypoxia. The effect of ATRA on angiogenesis in a rat intracerebral glioma model was also investigated, with the aim of revealing the effect of ATRA on glioma angiogenesis. In the present study, U-87 MG and SHG44 glioma cells were treated with ATRA at various concentrations (0, 5, 10, 20 and 40 μmol/l) under normoxia or hypoxia. Quantitative polymerase chain reaction and western blot analysis were used to investigate VEGF and HIF-1α mRNA and protein expression, respectively. An intracerebral glioma model was generated using intracerebral implantation of C6 glioma cells into rats. Tumor-bearing rats were treated with ATRA at different doses (0, 5 and 10 mg/kg/day) for two weeks, and immunohistochemical assays were performed to detect the cluster of differentiation 34-positive cells in order to evaluate the microvessel density (MVD) in each group. Following ATRA treatment, the expression of VEGF and HIF-1α was found to vary among the different concentration groups. In the glioma cells in the lower concentration groups (5 and 10 μmol/l ATRA), a significant increase in VEGF and HIF-1α expression was observed. Conversely, a significant decrease in VEGF and HIF-1α expression was found in the glioma cells in the high ATRA concentration group (40 μmol/l), compared with that in the cells in the control group. Furthermore, in the rat intracerebral glioma model, ATRA decreased glioma MVD, particularly in the high-dose group (10 mg/kg/day), compared with the control group. These results suggest that ATRA may exhibit a dose-dependent effect on glioma angiogenesis and may inhibit glioma angiogenesis *in vivo*.

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Introduction

Glioma is one of the most common types of brain tumor. Despite the development of current standard therapy, the prognosis of patients with malignant glioma is poor. All-trans retinoic acid (ATRA), a physiologically active derivative of vitamin A, is one of the most potent inducers of differentiation and has been extensively studied. The biological effects of ATRA are mediated through retinoic acid receptors (RARs) and retinoic X receptors. Several studies have shown that ATRA can induce differentiation and apoptosis in a variety of glioma cells (1,2), including glioma stem cells (3,4), a highly tumorigenic and therapy-resistant tumor subpopulation. ATRA can also induce growth arrest in glioma cells (5). Furthermore, studies have revealed that ATRA exhibits certain additional anti-glioma effects (6-11). Li *et al* (6) reported that ATRA enhanced the tumoricidal effect of suicide-gene therapy against medulloblastoma. Other studies have found that ATRA can significantly enhance the anti-tumor effect of certain chemoimmunotherapy and cytotoxic chemotherapy drugs, including interferon-γ and taxol, respectively, on glioma (7-11). These findings indicate that ATRA may have therapeutic potential in patients with glioma.

Angiogenesis is a complex biological process, which involves the degradation of the basement membrane and extracellular matrix, endothelial cell (EC) proliferation, migration and tube formation (12). Angiogenesis is mediated by various regulatory factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, hepatocyte growth factor (HGF) and angiopoietin-1 and -2. These regulatory factors are well-established angiogenic factors, and their biological effects are mediated through interaction

with their membrane receptors. Angiogenesis is important in numerous physiological processes, including embryo development, wound healing and normal growth (13). However, angiogenesis also has a key role in various pathological processes, such as inflammation (14), diabetic retinopathy (15) and the formation, development and recurrence of glioma (16). Therefore, anti-angiogenesis therapy has been developed as a novel therapeutic strategy for patients with glioma. Numerous studies have shown that anti-angiogenesis therapy can significantly inhibit glioma growth (17,18) and improve the outcome of patients with glioma (19,20). However, the effect of ATRA on glioma angiogenesis is yet to be elucidated.

VEGF induces EC proliferation, promotes cell migration and inhibits apoptosis. VEGF is well established as a key regulator of angiogenesis and plays an important role in the formation, development and recurrence of glioma (21-24). Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that is responsible for the induction of genes that regulate numerous biological processes, including angiogenesis (25). Furthermore, HIF-1α regulates various genes involved in the different stages of angiogenesis, including VEGF. VEGF is the most potent endothelial-specific mitogen and directly participates in angiogenesis by recruiting ECs and stimulating their proliferation (26).

The present study investigated the effect of ATRA on angiogenesis by detecting VEGF and HIF-1α expression in two glioma cell lines, U-87 MG (U87) and SHG44, under normoxia and hypoxia. The anti-angiogenic effect of ATRA in a rat intracerebral glioma model was also investigated. The results of this study are likely to provide an enhanced understanding of the mechanisms underlying the therapeutic effect of ATRA in malignant glioma.

Materials and methods

Materials. The U87 and SHG44 human glioma cell lines and the C6 rat glioma cell line were purchased from the Cell Resource Center, Chinese Academy of Sciences (Shanghai, China). A total of 30 male, specific pathogen-free, Sprague Dawley rats, weighing between 280 and 320 g, were obtained from the Laboratory Animal Center, Medical College of Xi'an Jiaotong University (Xi'an, China). All animal procedures were performed in accordance with the Guidance by the Research Ethics Committee of the Medical College of Xi'an Jiaotong University.

Cell culture. U87 cells were cultured in Dulbecco's Modified Eagle's Medium (Hyclone, Beijing, China), supplemented with 10% fetal bovine serum (FBS; Hyclone) in 5% CO_2 at 37˚C. SHG44 and C6 cells were cultured in RPMI-1640 (Hyclone), supplemented with 10% FBS in 5% CO_2 at 37˚C.

Quantitative polymerase chain reaction (qPCR) analysis. U87 and SHG44 cells were seeded onto six-well plates (Corning Inc., Lowell, MA, USA) at a density of $3x10^5$ cells/well. ATRA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored in light-protected vials at -20˚C as a stock solution. The stock solution was diluted to the desired concentration prior to use. All experiments were performed under low-light conditions to minimize ATRA photoisomerization. The cells were incubated in media containing various concentrations of ATRA (5, 10, 20 and 40 μmol/l) for 24 h under normoxia. The control group was treated with an equal volume of the solvent (DMSO) in the culture media.

$CoCl_2 \cdot 6H_2O$ (Sigma-Aldrich) was used to simulate hypoxia in order to investigate the effect of ATRA on VEGF and HIF-1α expression under hypoxic conditions. U87 and SHG44 cells were seeded in six-well plates at a density of $3x10^5$ cells/well and were incubated in media containing various concentrations of ATRA (5, 10, 20 and 40 μmol/l) and 100 μmol/l $CoCl_2$ for 24 h. The hypoxia control group was treated with an equal volume of the solvent (DMSO) and 100 μmol/l $CoCl_2$ in the culture media. The normoxia control group was solely treated with an equal volume of the solvent (DMSO).

The cells were lysed and the total RNA was isolated using the RNAfast200 kit (Shanghai Fastagen Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The RNA was reverse transcribed using PrimeScript™ RT Master Mix (Takara Bio Inc., Dalian, China). qPCR was performed using SYBR® Premix Ex Taq™II (Takara Bio Inc.) with a Bio-Rad IQ5 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed with Bio-Rad iQ5 software version 2.0. Gene expression was compared using the Δcycle threshold (ΔCt) method ($\Delta Ct=Ct_{Target}-Ct_{\beta\text{-actin}}$), where β-actin expression was used as an endogenous reference gene. The changes in target gene expression were evaluated using the $2^{-\Delta\Delta Ct}$ method (27). All the primers were designed and synthesized by Takara Bio Inc. (Table I).

Western blot analysis. U87 and SHG44 cells were cultured in 25-ml culture flasks (Corning Inc.) with ATRA at various concentrations under normoxia or hypoxia for 24 h, in accordance with the aforementioned methods. The cells were then harvested for the subsequent assays. The cells were washed twice with phosphate-buffered saline (PBS), then scraped on ice in 300 μl radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) with 1 mmol/l phenylmethylsulfonyl fluoride. The lysates were cleared of insoluble material using centrifugation and protein concentration was determined using a Bradford protein assay kit (Beyotime Institute of Biotechnology). The samples were boiled in 1X SDS-PAGE sample loading buffer, resolved using SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were probed with anti-VEGF (Epitomics, Burlingame, CA, USA) and anti-GAPDH (Bioworld Technology Inc., St. Louis Park, MN, USA) antibodies diluted 1:1,000 and 1:5,000, respectively. Membranes from the hypoxia groups were also probed with anti-HIF-1α antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA) diluted 1:1,000. Subsequent to washing in Tris-buffered saline containing 0.02% Tween 20, the membranes were incubated with a secondary polyclonal anti-rabbit immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) diluted 1:2,000. Membranes were developed using Super Signal™ West Pico chemiluminescent reagent (Pierce Biotechnology, Inc., Rockford, IL, USA).

Table I. Primer sequences for the quantitative polymerase chain reaction.

Gene	Sequence	Product length (bp)
HIF-1α	Forward, 5'-TCTGGGTTGAAACTCAAGCAACTG- 3' Reverse, 5'-CAACCGGTTTAAGGACACATTCTG-3'	150
VEGF	Forward, 5'-TCACAGGTACAGGGATGAGGACAC-3' Reverse, 5'-CAAAGCACAGCAATGTCCTGAAG-3'	72
β-actin	Forward, 5'-TGGCACCCAGCACAATGAA-3' Reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'	186

HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

Animal studies. A total of 10 μl cell suspension containing 1x10^6 C6 cells was injected at the coronal suture 3 mm away from the midline and at a depth of 5 mm into the right frontal lobe of the rats. Seven days after C6 cell injection, 30 tumor-bearing rats were randomly divided into three groups (10 rats/group). The low- and high-dose groups were treated with different doses of ATRA (5 and 10 mg/kg/day, respectively) diluted in corn oil. ATRA was administered using intraperitoneal injection between days 8 and 21. The control group was treated with an equal volume of solvent.

On day 22, after two weeks of treatment, all tumor-bearing rats were sacrificed. Brain and tumor samples were obtained, and frozen sections were prepared. Immunohistochemistry was performed to detect the cluster of differentiation (CD) 34-positive cells in order to evaluate the microvessel density (MVD) in each group using an immunohistochemical assay kit (Boster Biological Technology Co., Ltd., Wuhan, China). All procedures were performed according to the manufacturer's instructions. Briefly, the sections were fixed in 4% paraformaldehyde for 20 min and 3% hydrogen peroxide in methanol was then used to quench any endogenous peroxidase activity. Non-specific protein binding was blocked using 5% bovine serum albumin for 10 min at room temperature. The sections were incubated overnight with rabbit anti-CD34 antibody (Boster Biological Technology Co., Ltd.) diluted 1:100 at 4˚C. The negative control sections were incubated with PBS instead of the antibody. Subsequent to three washes in PBS, the sections were incubated in biotinylated anti-rabbit IgG antibody at 3˚C for 30 min. Following incubation, the sections were washed three times for 2 min in PBS and antibody location was determined using a 3,3'-diaminobenzidine substrate kit (Tiangen Biotech Co., Ltd., Beijing, China) for 5 min. Normal vascular endothelium was used as a positive control. MVD was determined as previously described by Weidner *et al* (28). Briefly, the tumor sections were scanned at low magnifications (x40 or x100) to determine the areas of most intense tumor angiogenesis, termed the 'hot spots'. Following 'hot spot' identification, the MVD was calculated by averaging the number of individual microvessels in five fields at high magnification (x400).

Statistical analysis. Values are presented as the mean ± standard deviation and data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to compare the groups and least significant difference tests were performed for further inter-group comparisons. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of ATRA on the VEGF and HIF-1α expression in glioma cells under normoxia. Following incubation with various concentrations of ATRA for 24 h, VEGF mRNA levels were observed to vary among the different groups. As shown in Fig. 1A, after 24 h the VEGF mRNA expression was observed to be significantly upregulated in the U87 and SHG44 glioma cells in the lower ATRA concentration groups (5 and 10 μmol/l), compared with that in the control group (P<0.01). Treatment with 5 μmol/l ATRA was found to increase VEGF mRNA expression ~2.4-fold in the U87 and 1.3-fold in the SHG44 glioma cells. Treatment with 10 μmol/l ATRA increased VEGF mRNA expression ~4.42-fold in the U87 and 2.24-fold in the SHG44 glioma cells. Conversely, following treatment with 40 μmol/l ATRA, the VEGF mRNA expression was observed to be significantly downregulated to 0.80- and 0.77-fold that in the corresponding control groups, in the U87 (P<0.01) and SHG44 (P<0.05) glioma cells, respectively. Furthermore, following treatment with 20 μmol/l ATRA, VEGF mRNA levels in the U87 glioma cells increased 1.53-fold relative to the levels in the control group (P<0.01); however, no significant difference was observed in the SHG44 glioma cells.

VEGF protein expression was analyzed using western blot analysis, as shown in Fig. 1B and C. In accordance with the changes in VEGF mRNA expression, treatment with lower concentrations of ATRA (5 and 10 μmol/l) was observed to upregulate VEGF protein expression in the U87 and SHG44 glioma cells. Furthermore, treatment with high ATRA concentrations (40 μmol/l) significantly inhibited VEGF expression in the glioma cell lines.

HIF-1α mRNA expression was also analyzed in the U87 and SHG44 glioma cells following ATRA treatment. As shown in Fig. 1D, qPCR revealed that, following treatment with lower concentrations of ATRA (5 and 10 μmol/l), HIF-1α mRNA expression was upregulated in the glioma cell lines. Treatment with 5 μmol/l ATRA was observed to upregulate HIF-1α mRNA expression ~1.41- and 1.24-fold in the U87 and SHG44 glioma cells, respectively. Furthermore, treatment with 10 μmol/l ATRA was found to upregulate HIF-1α mRNA expression ~2.2- and 2.5-fold in the U87 and SHG44 glioma

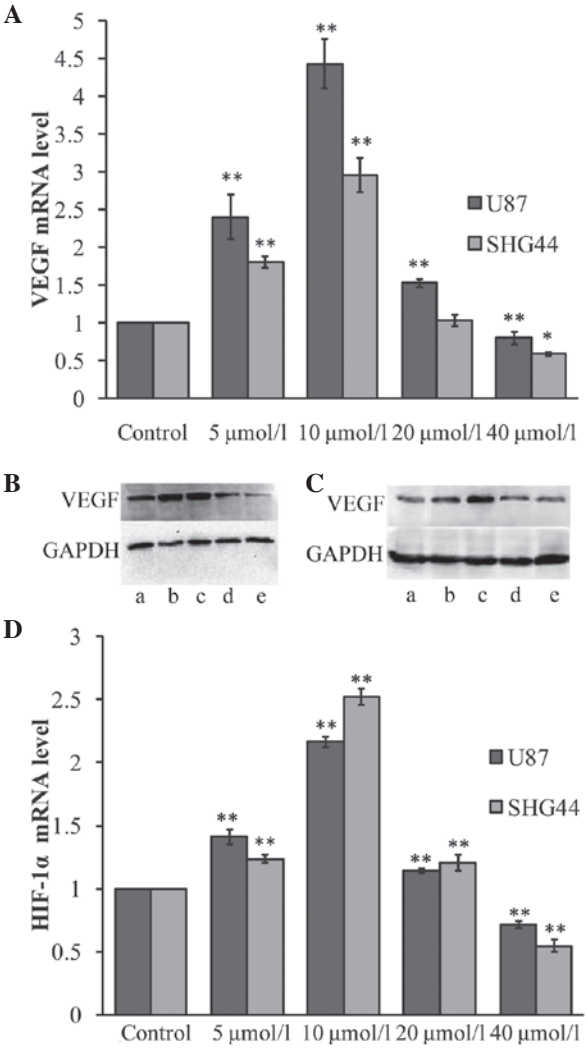

Figure 1. Effects of ATRA on VEGF and HIF-1α expression under normoxia. (A and D) Effects of ATRA on (A) VEGF and (D) HIF-1α mRNA expression. U87 and SHG44 glioma cells were treated with various concentrations of ATRA (5, 10, 20 and 40 μmol/l) for 24 h under normoxia. The control group was treated with an equal volume of solvent (dimethyl sulfoxide) in the culture media. VEGF and HIF-1α mRNA expression increased following treatment with 5 and 10 μmol/l ATRA in the two glioma cell lines (P<0.01 versus the control group). Following treatment with 40 μmol/l ATRA, the VEGF and HIF-1α mRNA expression was significantly downregulated (P<0.01 versus the control group). (B and C) Western blot analysis of VEGF protein expression in (B) U87 and (C) SHG44 glioma cells treated with various concentrations of ATRA. (a) Control; (b) 5, (c) 10, (d) 20 and (e) 40 μmol/l ATRA. *P<0.05 and **P<0.01 versus the control group. ATRA, all-trans retinoic acid; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α.

cells, respectively (P<0.01 versus the control group). However, treatment with 40 μmol/l ATRA was observed to significantly decrease HIF-1α mRNA expression in glioma cells to 0.71- and 0.55-fold that in the control group in the U87 and SHG44 glioma cells, respectively (P<0.01). HIF-1α protein is rapidly degraded under normoxic conditions; therefore, HIF-1α protein expression was not detected under normoxia.

Effects of ATRA on VEGF and HIF-1α expression in glioma cells under hypoxia. Hypoxia is one of the primary regulators of angiogenesis; therefore, the effects of ATRA on VEGF and HIF-1α expression was analyzed under hypoxia in the U87 and SHG44 cells. As shown in Fig. 2, hypoxia was observed to significantly upregulate VEGF protein and mRNA expression (P<0.01 versus the normoxia control group). However, hypoxia only increased HIF-1α protein expression, with HIF-1α mRNA expression unaffected by the hypoxia mimic (P>0.05).

Under hypoxia, the U87 and SHG44 glioma cells treated with various concentrations of ATRA exhibited a similar reaction to those under normoxia. VEGF and HIF-1α mRNA and protein expression was significantly upregulated by lower concentrations of ATRA (5 and 10 μmol/l), compared with the hypoxia control group (P<0.01). However, treatment with high concentrations of ATRA (40 μmol/l) was observed to decrease the expression of VEGF and HIF-1α (P<0.01).

Effect of ATRA on MVD in the rat intracerebral glioma model. In order to investigate the anti-angiogenic effect of ATRA on glioma *in vivo*, CD34 was examined using immunohistochemistry to assess glioma MVD in each group of tumor-bearing rats. As shown in Fig. 3, ATRA was found to significantly decrease the number of CD34-stained glioma microvessels, particularly at higher doses (10 mg/kg/day) (P<0.01 versus the control group). The average MVDs were 106.56±17.80, 68.04±11.95 and 33.66±12.05 in the control, low- (5 mg/kg/day) and high- (10 mg/kg/day) dose groups, respectively.

Discussion

It is well established that VEGF is a key regulator of angiogenesis and has an important role in the formation, development and recurrence of glioma. Several studies have reported that ATRA affects VEGF expression; however, the results are controversial (29-34). Reports have demonstrated that ATRA is capable of inhibiting VEGF expression in several cell types, including human gastric cancer (29), esophageal squamous cell carcinoma (30) and leukemia (31) cells. However, ATRA has been reported to have a stimulatory effect on VEGF expression in various other cell types, such as human umbilical vein ECs (32), human retinal pigment epithelial cells (33) and retinoblastoma Y78 cells (34). The only consensus regarding the effect of ATRA on VEGF expression is that the anti-leukemia effect of ATRA is exerted through the downregulation of VEGF (31,35-37). The contradictory results produced by these studies may be a consequence of differences in cell type, methodology, experimental conditions and the concentrations of ATRA. However, little is known concerning the effect of ATRA on VEGF expression in glioma.

In the present study, ATRA was observed to have a dose-dependent effect on VEGF expression in glioma cells. Lower concentrations of ATRA were found to significantly increase VEGF expression at the transcriptional and translational level in glioma cells. However, high concentrations of ATRA were observed to significantly decrease the expression of VEGF mRNA and protein. These converse effects have also been reported in ECs. Saito *et al* (32) reported that, at concentrations between 1 nmol/l and 1 μmol/l, ATRA induced the expression of VEGF and its receptor, VEGFR-2, in ECs. However, it has also been reported that 5 μmol/l ATRA decreases VEGF and VEGFR-2 expression, as well as Akt phosphorylation in ECs (38). In addition to the differences in

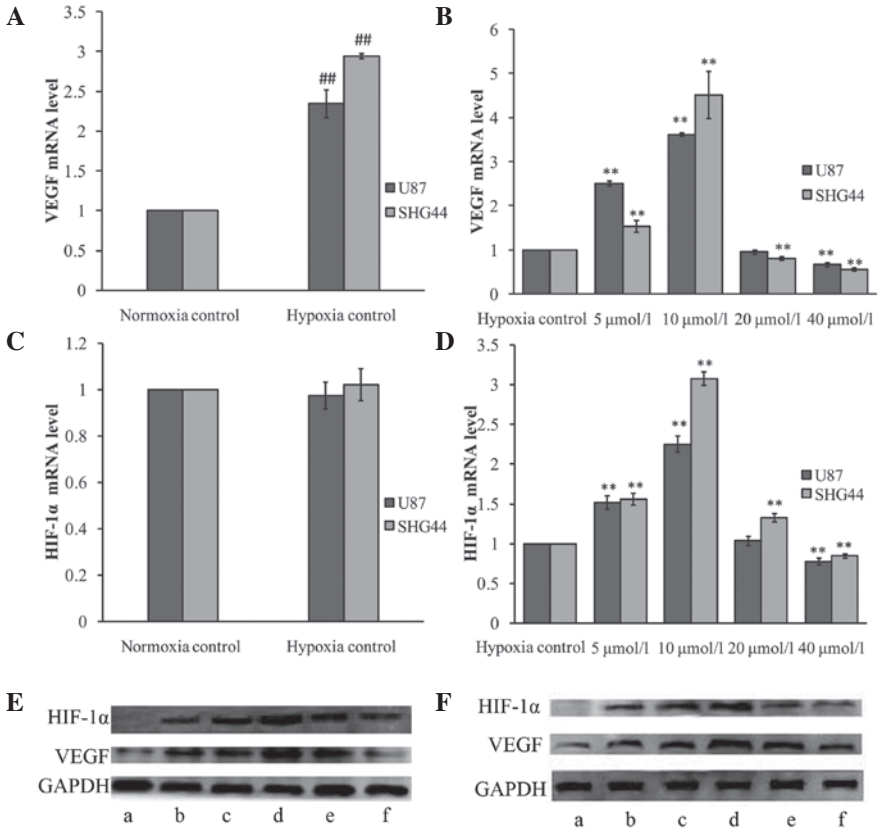

Figure 2. Effects of ATRA on VEGF and HIF-1α expression under hypoxia. (A and C) Effect of hypoxia mimic on (A) VEGF and (C) HIF-1α mRNA expression. Hypoxia was simulated using 100 μmol/l $CoCl_2$. The mRNA expression of VEGF, but not of HIF-1α, was induced by the hypoxia mimic. (B and D) Effects of ATRA on (B) VEGF and (D) HIF-1α mRNA expression under hypoxia. U87 and SHG44 glioma cells were treated with various concentrations of ATRA (5, 10, 20 and 40 μmol/l) for 24 h under hypoxia. The hypoxia control group was treated with an equal volume of solvent (dimethyl sulfoxide) and 100 μmol/l $CoCl_2$ in the culture media. The VEGF and HIF-1α mRNA expression increased following treatment with 5 and 10 μmol/l ATRA in the two glioma cell lines ($P<0.01$ versus the hypoxia control group). Following treatment with 40 μmol/l ATRA, the VEGF and HIF-1α mRNA expression was significantly downregulated ($P<0.01$ versus the hypoxia control group). (E and F) Western blot analysis of VEGF and HIF-1α protein expression in (E) U87 and (F) SHG44 glioma cells treated with various concentrations of ATRA. (a) Normoxia control; (b) hypoxia control; (c) 5, (d) 10, (e) 20 and (f) 40 μmol/l ATRA. $^{**}P<0.01$ versus the hypoxia control group; $^{\#\#}P<0.01$ versus the normoxia control group. ATRA, all-trans retinoic acid; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α.

methodology and experimental conditions in these two studies, the different concentrations of ATRA may have contributed to the contradictory results. The present study revealed that ATRA has a concentration-dependent effect on VEGF mRNA and protein expression in glioma cells.

The mechanism underlying the ATRA-induced regulation of VEGF expression is yet to be elucidated. Evidence suggests that ATRA increases the protein expression of specificity protein (Sp) 1 through post-transcriptional mechanisms, and that elevated Sp1 protein levels induce VEGF expression (34). It has also been shown that ATRA is capable of directly inducing VEGF expression through the RAR signaling pathway (32). However, it has additionally been suggested that ATRA may decrease VEGF gene transcription through a direct repeat 1 element located at the transcription initiation site (31). Furthermore, HIF-1α is well established as a key regulator of VEGF expression. Studies have indicated that ATRA regulates VEGF expression through the regulation of HIF-1α (39,40). Therefore, in the present study, the effect of ATRA on HIF-1α expression in glioma cells was investigated.

The effect of ATRA on HIF-1α mRNA expression was observed to be similar to that on VEGF expression. Lower concentrations of ATRA significantly increased HIF-1α expression and high concentrations of ATRA significantly decreased HIF-1α expression. HIF-1α protein is rapidly degraded under normoxic conditions and is undetectable; therefore, hypoxia was mimicked using $CoCl_2$, and the effect of ATRA on VEGF and HIF-1α expression, particularly HIF-1α protein expression, was investigated in glioma cells under hypoxia.

Hypoxia can induce angiogenesis in numerous physiological and pathological processes, primarily through the HIF pathway. In the present study, mimicking hypoxia was found to significantly increase HIF-1α protein, but not mRNA, expression. This phenomenon is associated with the mechanism underlying $CoCl_2$-mediated hypoxia mimicry (41). Briefly, cobalt displaces iron from the iron-binding site of the enzyme, thus interrupting with the degradation of the HIF-1α protein under normoxia. In the present study, following treatment with different concentrations of ATRA under hypoxia, similar dose-dependent changes in HIF1-α and VEGF protein expression were observed. Thus, there is reason to speculate that the regulation of VEGF expression by ATRA in glioma cells, may partially be through the regulation of HIF-1α expression.

The mechanism underlying the regulation of HIF-1α expression by ATRA remains unclear. It has been suggested

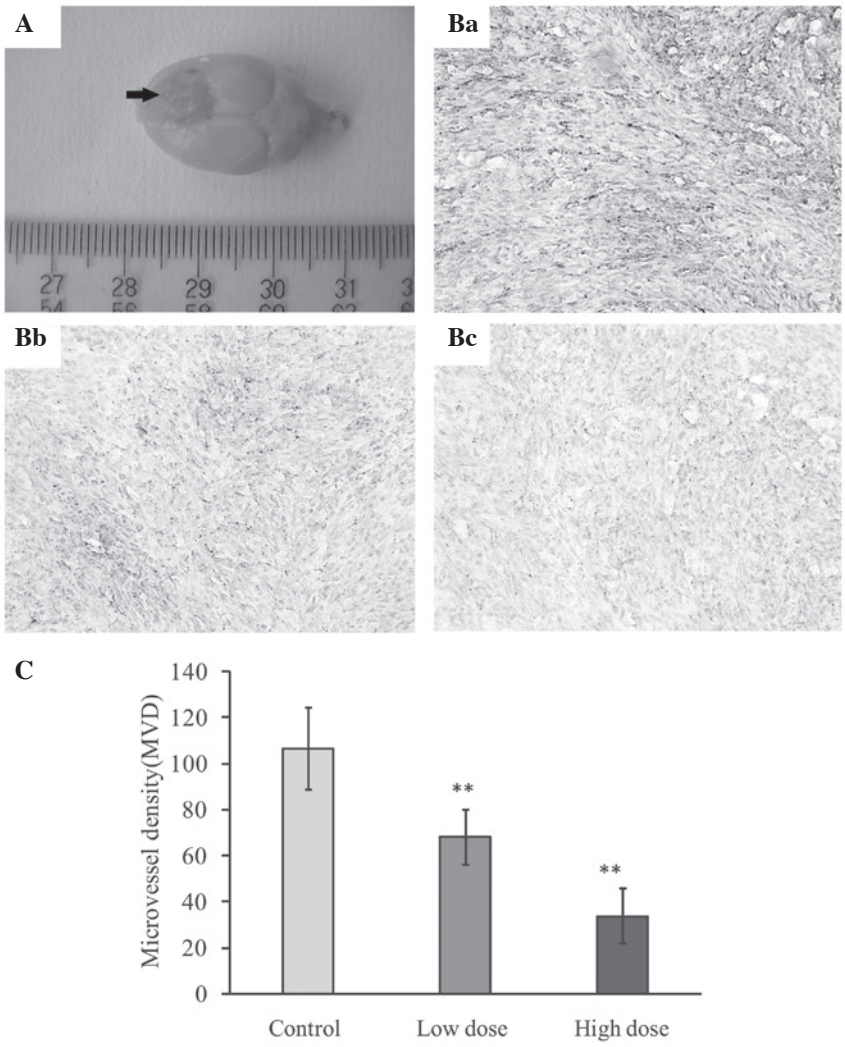

Figure 3. Effect of ATRA on MVD in a rat intracerebral glioma model. (A) Rat intracerebral glioma model (black arrow shows the tumor). (B) Immunohistochemical staining of cluster of differentiation 34 in the (a) control group, (b) low-dose group treated with 5 mg/kg/day ATRA and (c) high-dose group treated with 10 mg/kg/day ATRA (magnification, x100). (C) MVD in each group. Following treatment with ATRA, the glioma MVD was significantly decreased, particularly in the high-dose group. **P<0.01 versus the control group. ATRA, all-trans retinoic acid; MVD, microvessel density.

that the ATRA-induced upregulation of HIF-1α expression may involve the stabilization of HIF-1α mRNA (39). It has also been reported that ATRA can induce HIF-1α expression through intracrine prostaglandin E_2 signaling (40). However, the mechanisms underlying the inhibitory effect of high concentrations of ATRA on HIF-1α expression are yet to be elucidated, and further research is required.

In the present study, a rat intracerebral glioma model was generated in order to investigate the effect of ATRA on the inhibition of glioma angiogenesis *in vivo*. ATRA has been reported to inhibit angiogenesis in solid tumors, including esophageal squamous cell carcinoma (30). In the present study, ATRA was found to significantly decrease glioma MVD, particularly in the high-dose group.

The results of the present study suggest that, despite the increased expression of VEGF induced by lower concentrations of ATRA in glioma cells *in vitro*, ATRA has strong anti-angiogenic effects on glioma *in vivo*. However, the mechanisms underlying the anti-angiogenic effects of ATRA on glioma *in vivo* are yet to be fully elucidated. The findings of the present study suggest that the inhibitory effects of high concentrations of ATRA on VEGF and HIF-1α expression are likely to be associated with the anti-angiogenic mechanisms. Furthermore, ATRA has been reported to significantly inhibit the secretion and expression of HGF in glioma cells (42). HGF is an important pro-angiogenic cytokine; therefore, the inhibition of HGF may contribute to these anti-angiogenic effects *in vivo*. However, ATRA-mediated differentiation has also been shown to reduce angiogenesis in stem-like glioma cells *in vivo* (4). Therefore, the mechanisms underlying the anti-angiogenic effect of ATRA *in vivo* are likely to be complex and require further investigation.

VEGF has an important role in angiogenesis. The upregulation of VEGF expression in glioma cells induced by lower concentrations of ATRA may reduce its therapeutic effects on glioma *in vivo*. Therefore, as a therapeutic strategy, ATRA has certain limitation; the combination of ATRA with therapies targeting VEGF may have greater beneficial effects for the treatment of glioma.

In conclusion, in the present study, ATRA was found to have a concentration-dependent effect on the expression of VEGF and HIF-1α. Furthermore, ATRA was observed to

inhibit glioma angiogenesis *in vivo*; however, further research is required in order to reveal its mechanisms.

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