Aquaporin-4 upregulated expression in glioma tissue is a reaction to glioma-associated edema induced by vascular endothelial growth factor

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Abstract. Glioma-associated edema contributes significantly to morbidity and death in patients with glioma. It has been suggested that vascular endothelial growth factor (VEGF) and aquaporin-4 (AQP4) play important roles in glioma-associated edema. However, the effect of VEGF on AQP4 expression is not clear. In this study, AQP4 expression was assayed in cultured glioma cells that express different amounts of VEGF, and AQP4 expression, vessel permeability and water content were assayed in glioma xenografts that express different amounts of VEGF. No difference in AQP4 expression was found between glioma cells expressing different VEGF amounts *in vitro*. However, AQP4 expression was increased in glioma tissue with increased VEGF, vessel permeability and water content. In conclusion, VEGF does not directly affect AQP-4 expression. The redistribution of AQP4 in glioblastoma cells is a reaction to vasogenic edema induced by VEGF for the purpose of facilitating reabsorption of excess fluid. The pattern of AQP4 expression in glioma provides new insights into the molecular changes occurring in glioma-associated edema and may help plan future therapeutic strategies.

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Abbreviations: AQP4, aquaporin-4; VEGF, vascular endothelial growth factor; ZO, zonula occludens; JAM, junction associated molecules; ELISA, enzyme-linked immunosorbent assay; Q-PCR, quantitative polymerase chain reaction; HUVEC, human umbilical vein endothelial cells

Key words: glioma, edema, vascular endothelial growth factor, aquaporin-4

Introduction

Glioma progression can lead to glioma-associated brain edema, which is a significant source of morbidity and mortality (1). In-depth studies of molecular mechanisms of glioma-associated edema have implicated vascular endothelial growth factor (VEGF), aquaporin-4 (AQP4), cyclooxygenase-2, zonula occludens (ZO), occludins, claudins, and junction associated molecules (JAM) in the process (2-6). VEGF (one of the most important factors promoting angiogenesis) is also responsible for plasma extravasation leading to peritumoral tissue edema, increased vessel permeability, and increase in the water content of glioma tissue (7,8). Although some evidence is contradictory (9,10), most accumulating evidence suggests the involvement of AQPs in the dynamics of brain edema formation or resolution (11). Mou *et al* found that the degree of peritumoral edema correlates with peritumoral AQP4 protein expression and that AQP4 expression correlates with VEGF and HIF-1α expression (12). Another study showed that intracerebral VEGF injection dramatically upregulates AQP4 mRNA and protein in the perivascular space and glia limitans externa (13). Although there is a significant correlation between aquaporin-4 expression and the degree of cerebral edema, it is not clear whether increased aquaporin-4 expression enhances edema formation or clearance. The effects of VEGF on AQP4 expression may be important for understanding the molecular mechanism of edema. However, to our knowledge, there are no published reports on the effects of VEGF on AQP4 expression in glioma. The goal of the present study was to assess these effects and possibly provide a basis for developing novel therapeutic approaches for glioma-associated edema.

Materials and methods

Cell culture. Rat C6 glioma cells (Cell Biology Research Institute of Shanghai, Shanghai, China) and C6 cells with expression vectors containing antisense (C6/VEGF$^-$) VEGF164 cDNA or an empty vector (C6/vec) which were confirmed by assays for VEGF protein in cell culture supernatants and saved in our laboratory (7,8) were cultured in RPMI-1640 medium (1640M) (Invitrogen, Carlsbad, CA, USA) supplemented with fetal calf serum (10%).

For cell proliferation assay, $2x10^4$ cells were placed in a 6-well plate and were counted after 24, 48, 72, 96, 120 and 144 h culture by hemocytometer.

To measure VEGF secretion *in vitro*, $5x10^5$ cells were placed in 6-well plates and treated with serum-free 1640M. Medium was collected after 48 h of culturing. Debris was removed by centrifugation at 2000 x g for 5 min and supernatant was collected for enzyme-linked immunosorbent assay (ELISA). A commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to detect mouse VEGF according to the manufacturer's recommendations. Each experiment was performed a minimum of three times.

VEGF activity assay. Confluence (80-90%) of C6 cells, C6/vec cells and C6/VEGF$^-$ cells were treated with serum-free 1640M for 48 h. Media were collected and VEGF concentration were measured by ELISA. Human umbilical vein endothelial cells (HUVEC) ($2x10^4$, Cell Biology Research Institute of Shanghai) were placed in a 6-well plate and treated with 100 ng of VEGF secreted from three C6 cell lines. The HUVEC cell growth curve was monitored by cell count.

Xenograft glioma animals. Male 4-6-week-old BALB/c (nu/nu) mice (SLAC, Shanghai, China) (n=33) were randomized into three groups (n=11). Two individual clones of stable transfected C6 cells in the logarithmic growth phase were used for each construct (vector only, VEGF antisense), and the parental cell line was also studied. Two hundred microliters of cells (in serum-free medium with a final concentration of $7.5x10^6$ cells/ml) were injected subcutaneously into the right inguinal area of the mice. Anesthetized mice were sacrificed with decapitation and tumors were removed from the athymic (nu/nu) mice at 20 days post-implantation. The animals were sacrificed and the tumors were removed and then quickly frozen in liquid nitrogen for further analysis. 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.

Quantitative polymerase chain reaction (Q-PCR). Total RNA was isolated and cDNA was synthesized as has been described (7,8). The sequences of primer sets were VEGF, forward: 5'-CCCAAGCTTATGAACTTTCTGCTCTCTTG-3', reverse: 5'-CGCGGATCCTCACCGCCTTGGCTTGTC-3'; AQP4, forward: 5'-GCATGAATCCAGCTCGATCCTTTGG-3' revese: 5'-AATGGGTGGCAGGAAATCTGAGGC-3'; β-actin, forward: 5'-GAGGCATCCTGACCCTGAAG-3', reverse: 5'-CATCACAATGCCAGTGGTACG-3'. The calculation of expression levels of VEGF and AQP4 was normalized by β-actin.

Immunohistochemistry. To detect VEGF and AQP4 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 AQP4 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 (14). After incubation with anti-VEGF (1:150 dilution, United States Biological, Swampscott, MA, USA), and anti-AQP4 (1:150 dilution, Oncogene, Cambridge, 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). The slices were counterstained by hematoxylin and mounted by Permount (Maixin).

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 was collected 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 phenylmethanesulfonyl 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 Laboratories, Hercules, CA, USA), and was normalized to a concentration of 1 mg/ml. 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.

The water contents of tumor tissue assays. Referring to previously described studies (7,8), the water contents of the tumor samples were measured and taken to represent the degree of edema. Tumor tissues from the same sample which was also sampled for assays of VEGF expression were immediately weighed on an electronic analytical balance to obtain the wet weight (WW). The samples were then dried in a gravity oven at 100˚C for 24 h to obtain the dry weight (DW). Water content was expressed as a percentage of wet weight; the formula for calculation was (WW-DW)/WW x 100%.

Tumor vessel permeability. Tumor-bearing mice received a 0.1-ml/g i.v. injection of Evans blue dye (1% in saline; Sigma-Aldrich, St. Louis, MO, USA). After 6 h the animals were sacrificed and Evans blue was extracted from tumor as described (7). Briefly, tumors were removed and homogenized with 3 ml of *N,N*-dimethylformamide (Sigma-Aldrich), and incubated at 57˚C for 12 h. The solutions were vortexed, then 2 ml of 1 N hydrochloric acid (HCl) were added, and the solutions were vortexed again, and then centrifuged at 2,500 rpm for 15 min. Supernatant was collected and measured at 620 nm with a spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Concentrations were calculated by using a standard curve for Evans blue dye.

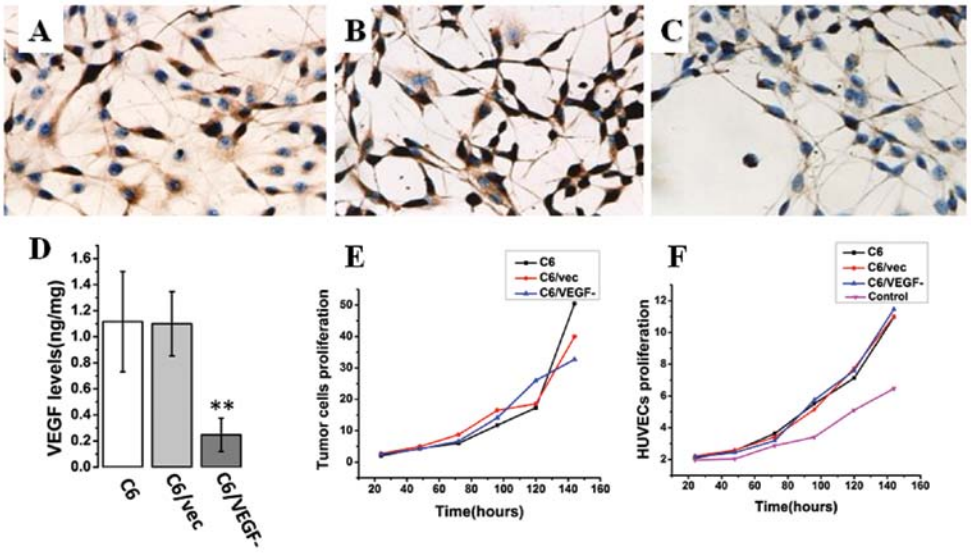

Figure 1. Expression of VEGF in glioma cells which express differential levels of VEGF *in vitro*. Immunocytochemical staining with antibody against VEGF antigen in C6 cells (A), C6/vec cells (B) and C6/VEGF$^-$ cells (C) after 48-h serum deprivation. (D) Enzyme linked immunosorbent assay (ELISA) of VEGF levels in condition media of C6 cells, C6/vec cells /and C6/VEGF$^-$ cells after 48-h serum deprivation. (E) Time course of cell proliferation of C6 cells (black), C6/vec cells (red) and C6/VEGF$^-$ cells (blue). (F) Time course of HUVEC cell growth curve with treatment of VEGF released from C6 cells (black), C6/vec cells (red), C6/VEGF$^-$ cells (blue) and non-treated (purple). *P<0.05; n=4-6.

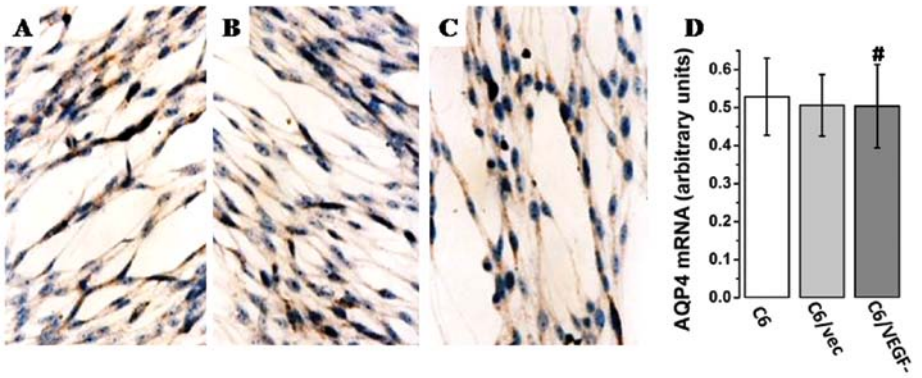

Figure 2. AQP4 expression in glioma cells which express differential levels of VEGF *in vitro*. Immunocytochemical staining with antibody against AQP4 antigen in C6 cells (A), C6/vec cells (B) and C6/VEGF$^-$ cells (C). The expression levels of AQP4 mRNA were assessed by quantitative PCR (D). $^\#$P>0.05; n=4-6.

Protein analysis. To analyze AQP4 expression *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 AQP4 (1:100), and β-actin (1:2000, Neomarker, Fremont, CA, USA). Bands were visualized using an electrochemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis. SPSS 12.0 software (SPSS, Chicago, IL, USA) was applied for statistical data analysis. Data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparisons to the control groups. Differences were considered significant at P<0.05.

Results

Expression of VEGF in glioma cells in vitro. Immunostaining with VEGF antibody showed that endogenous VEGF level was lower in C6/VEGF$^-$ cells than in C6/vec and C6 cells after 48 h of serum deprivation (Fig. 1A-C). Similarly, the level of VEGF protein in the medium from C6/VEGF$^-$ cells was significantly lower than that from C6/vec and C6 cells (Fig. 1D). To investigate the effect of antisense VEGF on tumor cell proliferation, the number of C6/VEGF$^-$, C6/vec, and C6 cells, respectively, placed into 6-well plates ($2x10^4$ cells/well) were counted after 24, 48, 72, 96, 120 and 144 h in culture. Proliferation of C6/VEGF$^-$ cells was found to be slower than that of the two control cells (C6/vec and C6; Fig. 1E), the morphology of all three cell lines was similar and remained unchanged (data not shown). However, the biological activity of VEGF released from different cell lines was not altered. Monitoring the growth of HUVEC cells treated with the same amount of VEGF secreted

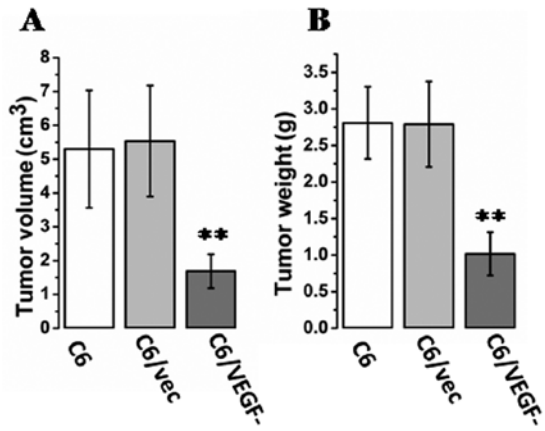

Figure 3. Characterization of xenograft glioma animals which express differential levels of VEGF. Analyses of tumor volume (A) and tumor weight (B) in C6 cell mice, C6/vec cells and C6/VEGF$^-$ cell mice. **P<0.01; n=7.

from our three C6 cell lines revealed similar levels of HUVEC cell growth, regardless of the source of VEGF, and slower growth in the absence of VEGF (Fig. 1F).

Effect of glioma-derived VEGF on AQP4 expression in glioma cells in vitro. AQP4 expression in the three cell lines was assayed by RT-PCR and immunostaining. Similar AQP4 mRNA levels (Fig. 2A) were found in all three cell lines. In addition, the intensity of AQP4 immunoreactivity was similar in all lines after 48 h of serum deprivation (Fig. 2B-D). Thus, VEGF does not appear to have a direct role in AQP4 expression in glioma tumor cells.

Effect of VEGF on tumorigenesis. To evaluate the possible role of VEGF in tumorigenesis, mice were injected with glioma cells (C6, C6/vec, or C6/VEGF$^-$ cells) directly into the right inguinal area, and tumor size was measured. At 20 days after inoculation, tumor size (Fig. 3A) and tumor weight (Fig. 3B) were notably smaller in C6/VEGF$^-$ mice.

Expression of VEGF in tumors and the water content of tumor tissue. To confirm the expression of VEGF *in vivo* as well as *in vitro*, VEGF levels were determined in genetically modified C6 cells by RT-PCR and ELISA. ELISA analysis (Fig. 4A) and RT-PCR (Fig. 4B) showed markedly lower level of VEGF mRNA and protein, respectively, in tumors from C6/VEGF$^-$ mice than tumors from C6/vec and C6 mice. A common feature of malignant brain tumors is increased capillary permeability leading to edema. Assay of tumor water content showed that C6/VEGF tumors had a lower water content than either of the two control tumors (C6/vec and C6) (Fig. 4C). To confirm that the edema was attributable to vascular hyperpermeability, vascular extravasation was examined using a dye tracer. Vascular leakage was markedly reduced in the C6/VEGF$^-$ tumors (Fig. 4D). Also, Pearson's correlation analysis found a correlation between water content and VEGF expression (Pearson's correlation, r=0.946 P=0.00).

Expression of AQP4 in tumors. Levels of AQP-4 appeared to be lower in C6/VEGF$^-$ tumors compared with C6G, C6/vecG tumors by immunohistochemistry (Fig. 5A-C), and western blot analysis (Fig. 5D and E), and Pearson's correlation analysis show that AQP4 expression paralleled the level of VEGF expression (r=0.883, P=0.00) and the water content (r=0.912, P=0.00) of glioma tissue. Thus, aquaporin-4 expression in glioma tissue is suggested to be a reaction to glioma-associated edema induced by VEGF.

Discussion

Aquaporins (AQPs) are a family of water channel proteins that facilitate the flux of water through plasma membranes. AQP4, a mercury-insensitive water channel protein, is abundant in the central nervous system. It is localized in the blood-brain barrier around blood vessels and luminal membrane of ependymal cells, and its distribution in high density astrocytic foot processes is polarized. AQP4 is speculated to maintain the homeostasis of intracellular and extracellular water in the brain (10,11,15). In addition, chemotherapy and radiotherapy for glioblastoma multiforme is reported to downregulate AQP4 expression, restoring its perivascular rearrangement and suggesting the potential role of AQP4 in the resolution of brain edema (16). Recent studies show that AQP4 is involved in cell migration and cytoskeleton organization (15,17). Taken together, these findings suggest that AQP4 has a critical role in glioma malignancy.

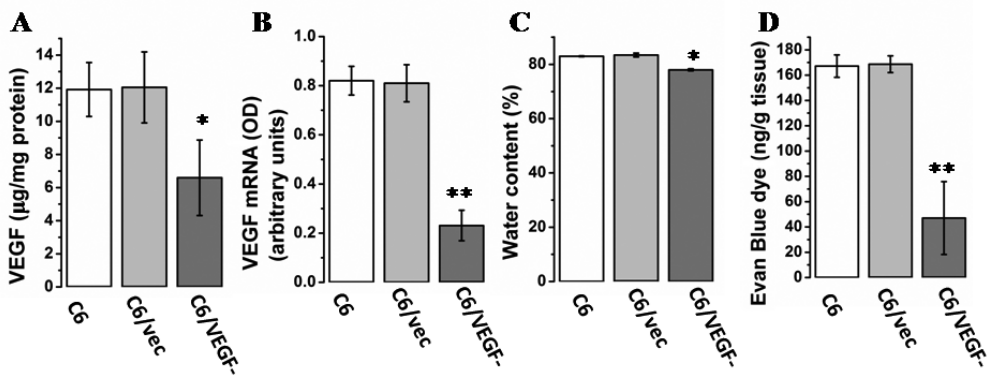

Figure 4. Expression of VEGF in tumors, vascular leakage and the water content of tumor tissue. VEGF protein and mRNA in tumor tissue were assessed by (A) ELISA and (B) Q-PCR. (C) Measurement of water content in tumor tissues. (D) Quantitative vascular leakage assessed by Evan blue dye tracer. *P<0.05; **P<0.01; n=4-6.

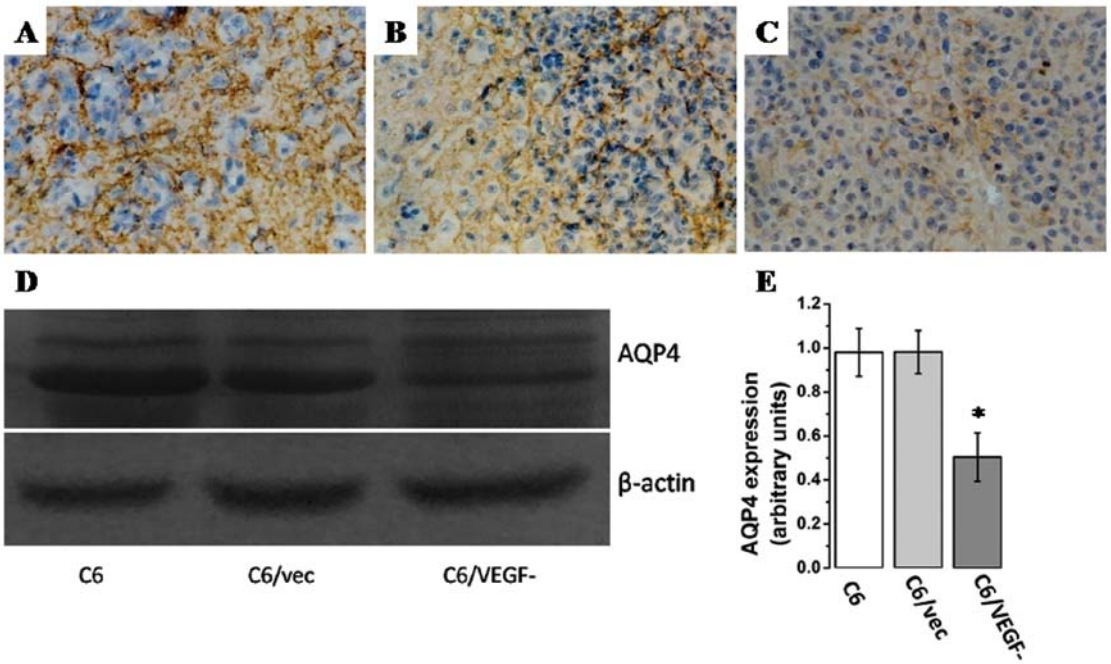

Figure 5. AQP4 analysis in xenograft glioma tissue which express differential levels of VEGF. Immunocytochemical staining with antibody against AQP4 antigen in C6 cell glioma (A), C6/vec cell glioma (B) and C6/VEGF$^-$ cell glioma (C); AQP4 western blot analysis (D); quantitative expression levels of AQP4 (E). $^*P<0.05$; n=4.

Breakdown of the blood-brain barrier (BBB) has been linked to upregulation of AQP4 expression. The increased AQP4 expression in high grade astrocytomas may facilitate the flow of edema fluid (18). The pattern of AQP4 expression in human gliomas, AQP4 overexpression in glioma cells, and AQP4 localization on astrocytic end-feet are associated with disturbance of the blood-brain barrier (19). The redistribution of AQP4 in glioblastoma cells is believed to facilitate reabsorption of excess fluid and to be a reaction to vasogenic edema stemming from the breakdown of the BBB (20).

Mou *et al* hypothesized that AQP4 is positively regulated by VEGF (12). Rite *et al* (13) found that intracerebral injection of VEGF induces an increase in AQP4, but in our study, VEGF did not directly affect AQP-4 expression. The most important factor regulating the function and expression of AQP4 is osmotic pressure. Studies have shown that VEGF may alter vascular permeability and affect osmotic pressure changes (21). VEGF can increase neovascular permeability and promote the extravasation of plasma protein and fibrinogen into intracellular spaces. In glioma, VEGF is one of the most important factors promoting angiogenesis in the tumor. With growth of the tumor, increase in vascular permeability due to neovascularization causes extensive damage to the BBB integrity, and a large number of macromolecules in plasma enter the interstitial space, where they produce an obvious change in osmotic pressure. Therefore, VEGF is an important factor affecting osmotic pressure within glioma tissue. Therefore, although AQP4 was associated with brain edema formation, we presume that upregulated expression and redistribution of AQP4 in glioblastoma cells is a reaction to VEGF-induced vasogenic edema and a response that ameliorates or prevents cytotoxic brain edema by facilitating reabsorption of excess fluid.

In summary, VEGF does not directly affect AQP-4 expression. The redistribution of AQP4 in glioblastoma cells is a reaction to VEGF-induced vasogenic edema and facilitates reabsorption of excess fluid. AQP4 induction might be a promising approach in vasogenic brain edema prevention and treatment. Further studies are needed to understand its functional role.

Acknowledgements

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