Expression of Vascular Endothelial Growth Factor in Human Meningiomas and Peritumoral Brain Areas

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Abstract. Vascular endothelial growth factor (VEGF) is a regulator of angiogenesis, vasculogenesis, and vascular permeability. Recent reports suggest that VEGF may play a critical role in formation of peritumoral brain edema (PTBE) associated with meningiomas. While VEGF expression has been shown in meningiomas, studies have not focused on VEGF in the adjacent peritumoral brain regions. The present study examined the protein and gene expression of VEGF in human meningiomas and peritumoral brain areas. Biopsies were obtained from 37 patients. Immunohistochemical staining and immunoblotting were performed to detect the expression of VEGF protein. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the presence and quantity of VEGF mRNA. The extent of PTBE was estimated as an edema index (EI) based on preoperative magnetic resonance imaging. In meningiomas, western blot and RT-PCR results were congruent and the expression of both protein and mRNA had a significant correlation with EI. However, in peritumoral areas, western blot results were not consistent with the RT-PCR results. Protein results showed high correlation with EI, but mRNA was almost undetectable. In VEGF-positive cases, a decreasing gradient of VEGF protein expression was observed with increasing distance from tumors. These data suggest that peritumoral tissue does not produce VEGF and that VEGF protein levels in peritumoral tissues have a high correlation with EI. We conclude that VEGF macromolecules are secreted by the tumor tissue and enter peritumoral normal brain tissue to induce edema.

Keywords: meningiomas, vascular endothelial growth factor, peritumoral brain edema

Introduction

Peritumoral brain edema (PTBE) is one of the most serious complications in the management of intracranial meningiomas and plays an important role in the pathophysiology and clinical profiles of meningiomas [1]. The severity of PTBE may limit operative exposure and increase the difficulty and complexity of intraoperative procedures. The development of PTBE occurs in approximately 60% of meningiomas [8,36]. Nevertheless, the pathophysiological mechanism remains unclear. Various causative factors have been discussed, including patient age [21], gender [21], tumor size [5,7-9,25,29,30], tumor location [7-9,15,25,29,30], histological differentiation [7,18,19,25,29,30,33], arterial blood supply to the tumor [6,10,15,25,38], intratumoral venous congestion [41], relation to hormone receptors [2,33], brain ischemia caused by tumor compression [42], and stasis induced by compromised venous drainage resulting from tumor mass effects [5,13,22].

VEGF has been implicated in many studies as a critical factor in the formation of PTBE associated with meningiomas [20,26,34,35,39,40]. VEGF is a specific mitogen of endothelial cells in vitro [27,28] and a potent promoter of angiogenesis in vivo [46]. Furthermore, VEGF has an effect on vascular permeability that is similar to vascular permeability factor (VPF) [27,28]. VEGF is a homodimeric glycoprotein of 36-46 kDa. Molecular cloning of the cDNA has revealed 4 types of VEGF in...
human cells. These forms result from alternative splicing of mRNA [23,44] and have been termed VEGF121, VEGF165, VEGF189, and VEGF206. The best characterized and most common form is VEGF165, which is a secretable and heparin-binding isoform [17].

Although VEGF has been hypothesized to be secreted by the tumor tissues and to enter peritumoral normal brain tissue [47], no studies have been performed to test whether it is expressed in the adjacent peritumoral brain region. This study examines the pattern of VEGF expression in meningioma tissue and peritumoral brain areas.

Materials and Methods

Sample collection. Thirty-seven patients with 40 intracranial supratentorial meningiomas were recruited for the study. They underwent surgery at our department between July 2005 and June 2006. The study was approved by the Ethical Committee of the Faculty of Medicine of Jinling Hospital.

After obtaining the patients’ informed consent, we resected the tumors and removed some peritumoral brain tissue. Patients consisted of 13 males and 24 females (mean age, 56.5 yr; range, 25 to 75 yr). Magnetic resonance imaging (MRI) was performed in all cases. The presence of PTBE was determined and quantified based on the MRI images. Using intraoperative navigation, tissue samples were collected during surgery from two sites: (a) tumor tissue, from the main bulk of the tumor tissue; and (b) peritumoral brain tissue, from the brain tissue adjacent to the tumor and involved in edema. The sample size was about 5 mm³. Normal brain white matter was obtained from five patients with intractable epilepsy treated by partial temporal lobectomy after obtaining the patients’ informed consent. Tissue samples were fixed in 10% formalin and embedded in paraffin for histological examinations. For western blotting and RT-PCR, materials were snap-frozen in liquid nitrogen and kept at –80°C until use. All tumors were reviewed independently by two neuropathologists and diagnosed according to the revised WHO classification of brain tumors.

The cases included 14 transitional, 11 meningothelial, 6 fibrous, 2 microcystic, 1 psammomatous, 1 angiomatous, 1 secretory, 3 atypical, and 1 malignant (anaplastic) meningioma. Tumors were further classified by location: 17 convex, 9 parasagittal, 7 falx, 3 middle fossa, 2 sphenoid ridge, and 2 frontal base.

Evaluation of magnetic resonance imaging. Tumor and edema volumes were approximated from MRI scans, similar to methods of other authors [6,12,25,41]. Tumor volume was estimated on T1-weighted scans using gadolinium-diethylene-triamine penta-acetic acid (0.1 mmol/kg) enhancement. PTBE was evaluated on T2-weighted or fluid-attenuated inversion recovery scans. Maximum perpendicular diameters (radius: a,b) were measured from the axial images and the extent in the coronal direction (radius: c) was estimated from the number of axial images displaying the structure multiplied by slice thickness. The total volume of the tumor \(V_{\text{tumor}}\) and the volume of the hyperintensity area on T2-weighted images \(V_{\text{T2-high}}\) were then approximated using the formula for a spheroid \(V=\frac{4\pi abc}{3}\). The relation of the tumor to PTBE volume (edema index [EI]) was defined using the formula \(EI = \frac{V_{\text{T2-high}} - V_{\text{tumor}}}{V_{\text{tumor}}}\). The formula yielded a value of 0 when no brain edema was found. The grading of edema severity according to EI was performed as follows: grade 0, no edema or negligible edema (EI <0.1); grade 1, mild edema (0.1 <EI <1.0); grade 2, moderate edema (1.0 <EI <2.0); grade 3, severe edema (EI >2) [32].

Immunocytochemistry. Tissue was fixed with 10% neutral buffered formalin and embedded in paraffin. Tissue sections (4 µm) were used for immunohistochemical staining, which was performed with an anti-VEGF polyclonal antibody (diluted 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were incubated with the diluted antibody overnight at 4°C, washed, and blocked with 1.6% H₂O₂ in phosphate-buffered saline (PBS) for 10 min. After washing with PBS, each section was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:500, Santa Cruz Biotechnology) for 60 min at room temperature. Diaminobenzidine (DAB) was used as the chromogen and counterstaining was done with hematoxylin. Negative controls consisted of PBS instead of primary antibodies. Evaluation of the immunohistochemical staining was carried out independently by two observers. Specimen sections and controls were rated on an arbitrary 1-to-4-point scale based on staining intensity, which was considered to be VEGF immunoreactivity. The ratings were arbitrarily designated as follows: no staining = 1, mild staining = 2, moderate staining = 3, and intense staining = 4. Staining intensity scores were the mean values assigned by observers.

Western blot analysis. The frozen tumor and brain tissue was mechanically lysed in 20 mM Tris, pH 7.6, which contained 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.11 IU/ml aprotinin (all from Sigma-Aldrich, St. Louis, MO, USA). Lysates were centrifuged at 12,000 g for 20 min at 4°C. The protein concentration was estimated by the Bradford method using a protein assay kit (Nanjing Jincheng Bioengineering Institute, Nanjing, China). The samples (60 µg per lane) were separated by 8% SDS-PAGE and electro-transferred onto a polyvinylidene-difluoride (PVDF) membrane (Bio-Rad Lab, Hercules, CA, USA). The membrane was blocked with 5% skim milk for 2 hr at room temperature and incubated overnight at 4°C with primary antibodies directed against the VEGF protein in PBS-Tween 20 (PBST) at a dilution of 1:200; β-actin (diluted 1:8,000 in PBST, Sigma-Aldrich) was used as a loading control. After the membrane was washed 6 times for 10 min each in PBST, it was incubated in the appropriate HRP-conjugated secondary antibody (diluted 1:400 in PBST) for 2 hr. Blotted protein bands were visualized by enhanced chemiluminescence (ECL) western blot detection reagents (Amersham, Arlington Heights, IL, USA) and were...
exposed to X-ray film. Developed films were digitized using an Epson Perfection 2480 scanner (Seiko Corp, Nagano, Japan). Optical densities were obtained using Glyko Bandscan software (Glyko, Novato, CA, USA) and VEGF expression levels were normalized to β-actin. All experiments were repeated at least 3 times.

**VEGF mRNA expression determined by RT-PCR.** Total RNA was extracted with TriPure Reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer’s instructions. The cDNA synthesis from the isolated RNA was performed using a reverse transcriptional system. Briefly, 4 µg of total RNA was reversely transcribed using 0.5 µg oligo(dT)15 and incubated with 15 U Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) (all from Promega, Madison, WI, USA). The cDNA was amplified by PCR using specific oligonucleotide primers (Table 1). The amplified fragments were detected by agarose gel electrophoresis and visualized by ethidium bromide staining. The intensity of the bands was quantified using Glyko Bandscan software. The positive control, β-actin mRNA, was detected in all samples; VEGF/β-actin product ratios were calculated and used as an index of VEGF mRNA expression. All experiments were repeated at least 3 times.

**Statistical Analysis.** Results are expressed as mean ± SE unless otherwise noted. Student’s t-test was used for comparisons of VEGF/β-actin ratio between the PTBE-negligible group and the PTBE-severe group. The relationship between VEGF/β-actin ratio and EI was evaluated using Pearson’s correlation coefficient. Calculations were performed using a statistical analysis system (SPSS-System). Differences were considered significant when p was <0.05.

### Table 1. PCR primer sequences.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Cycles</th>
</tr>
</thead>
</table>
| VEGF   | Sense: 5'-TCGGGCCTCCGAACCATGA-3'  
         | Anti-sense: 5'-CCTGGTGAGAGATCTGGTTC-3' | 516  
         | 648                         | 57  
         | 35                           | |
| β-actin| Sense: 5'-TACTGCCATCCCAATCGAGACC-3'  
         | Anti-sense: 5'-AATGCTTTCTCCGCTCTG-3' | 430  
         |                      | 57  
         | 30                           | |

### Table 2. Protein and mRNA ratio of VEGF in different brain regions (mean ± SE).

<table>
<thead>
<tr>
<th></th>
<th>Intratumoral tissues</th>
<th>Peritumoral tissues</th>
<th>Normal brain tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean protein ratio (VEGF/β-actin)</td>
<td>0.38±0.08</td>
<td>0.20±0.03</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>Mean mRNA ratio (VEGF/β-actin)</td>
<td>0.48±0.11</td>
<td>0.06±0.02</td>
<td>0.05±0.01</td>
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### Results

**Relationship between intratumoral VEGF expression and PTBE.** For assessment of the localization of VEGF in meningiomas, an immunohistochemical study of VEGF was performed. We observed that positive VEGF reactivity occurred mainly in the cytoplasm of tumor cells (Fig. 1A) and in the endothelium of tumor vessels (Fig. 1B).

To quantify the expression of VEGF, western blots were performed to assess VEGF protein levels in the tumors. Two obvious protein bands were detected in all PTBE-positive tumor samples. The major form migrated at approximately 23 kDa, and a minor band at 20 kDa (Fig. 2B). For all cases, immunohistochemical and immunoblotting results were congruent. Samples that were negative immunohistochemically did not show any detectable band by western blot analysis, although western blots showed a low level of VEGF protein in the PTBE-negligible group (Fig. 2B,C). To quantify the expression of VEGF, the intensity of the two bands was summed. Compared to PTBE-negligible meningiomas, tumors with severe PTBE revealed higher VEGF expression (VEGF relative intensity = 0.08 vs VEGF relative intensity = 0.53; p <0.01). Furthermore, EI correlated significantly...
with the intensity of VEGF protein expression ($r = 0.892$, $p < 0.01$).

Fig. 3B shows VEGF mRNA expression in meningiomas. Two obvious mRNA bands were also detected. The major fragment was 648 bp; the second major band was 516 bp (Fig. 3B). In all cases, RT-PCR and immunohistochemical results were consistent. RT-PCR analysis showed that VEGF mRNA was expressed at a low level in the PTBE-negligible group, whereas VEGF mRNA was expressed at a high level in the PTBE-severe group. To quantitatively compare the expression of VEGF, the intensity of the two bands was also summed. There was a significant difference between the severe group and the negligible group (VEGF Ratio = 0.07 vs VEGF Ratio = 0.68; $p < 0.01$). EI correlated significantly with the intensity of VEGF mRNA ($r = 0.875$, $p < 0.01$).

**Relationship between peritumoral VEGF expression and PTBE.** To assess of the localization and expression of VEGF in peritumoral brain tissues, an immunohistochemical study for VEGF was performed. The positive VEGF reactivity was mainly in the interstitial tissue (Fig. 1C).

Western blotting was performed to quantify VEGF protein levels in peritumoral brain tissues. Two clear protein bands were detected. The major form migrated at approximately 23 kDa, and a second major band was found 20 kDa (Fig. 2A). For all cases, the immunohistochemical and immunoblotting results were congruent. Western blot analysis showed a low level of VEGF protein in the PTBE-negligible group (Fig. 2A, C). Compared to the PTBE-negligible group, the severe PTBE group revealed significantly higher VEGF expression (VEGF relative intensity = 0.04 vs VEGF relative intensity = 0.35; $p < 0.01$). Moreover, the EI correlated significantly with the intensity of VEGF protein expression ($r = 0.912$, $p < 0.01$). Fig. 3A shows VEGF mRNA expression in peritumoral brain tissues. VEGF mRNA was practically undetectable. Fig. 3C shows that there was no significant difference between the PTBE-negligible group and the severe PTBE group.

**VEGF expression in intratumoral, peritumoral, and normal tissues.** To highlight the relationship of VEGF expression in intratumoral, peritumoral, and normal tissues, we calculated the mean protein ratio and mean mRNA ratio in 3 different regions.
for VEGF-positive cases (Table 2). Twenty-eight meningiomas expressed VEGF protein, but only 21 peritumoral brain tissues had detectable VEGF protein. A decreasing gradient of VEGF protein expression was observed with increasing distance from tumors. Consistent with the protein expression, 28 meningioma tumor tissues were detected with VEGF mRNA. However, VEGF mRNA was almost undetectable in peritumoral brain tissues. This value was similar to the value found in normal brain tissue (Table 2).

Discussion

The most important findings of this study are that (1) in VEGF-positive cases, VEGF protein expression decreases with increasing distance away from tumors; (2) VEGF mRNA could only be detected in tumors; and (3) in the peritumoral areas, VEGF protein levels showed high correlation with EI.

Meningiomas grow extra-axially and are separated from the brain parenchyma by the
arachnoid, the subarachnoid space, and the pia mater. Part of the blood–brain barrier, the arachnoid, is impermeable to fluids. In contrast, the pia mater shows high permeability to water and electrolytes, but is far less permeable to macromolecules, such as proteins in edema fluid [18]. In addition, the cerebral cortex, because of its intricately interwoven cellular processes, poses a structural hindrance that is almost impossible for vasogenic edema to overcome. Thus, the reasons for the formation of PTBE are complicated.

Various groups have proposed hypotheses to explain the generation of edema, including ischemic processes [42], venous congestion due to tumor compression [5,13,22], a secretory–excretory phenomenon [20,26,34,35,39,40], and a hydrodynamic process [3]. Moreover, controversy persists about the importance of tumor size [5,7-9,25,29,30,47], location [7-9,15,25,29,30], and histology [7,18,19,25,29,30,33] for the genesis of edema. There is no consensus regarding PTBE pathogenesis.

However, VEGF has been implicated as a critical factor in the formation of PTBE [20,26,34,35,39,40] based on the following findings: First, it is widely accepted that brain edema accompanying meningiomas arises from a vasogenic rather than a cytotoxic origin. Electron microscopy has revealed similarities between meningioma-associated edema and experimentally induced vasogenic edema [18]. Second, it is widely accepted that VEGF is a potent inducer of tumor angiogenesis and vascular permeability. VEGF is 1,000-fold more potent than histamine in inducing capillary permeability [11]. Third, Dvorak et al [16] have found that VEGF is synthesized in tumor cells and accumulates in the endothelium of tumor vessels. Meningiomas with strong VEGF staining demonstrated a significantly higher edema incidence and edema index than VEGF-negative cases [4,20]. Other authors noted increased VEGF mRNA-expression in edema-associated meningiomas compared to cases without edema [26]. Fourth, a previously published study demonstrated strong correlation between the adherence of the tumor to the surrounding brain tissue and the occurrence of edema [24]. Another study found a qualitative and quantitative correlation between pial blush and PTBE in meningiomas [6]. Paek et al [12] found that MMP-2 and MMP-9, which are well-known proteolytic enzymes that break down the basal membrane and connective tissue, were strongly correlated with the presence of PTBE in meningiomas. These studies showed disruption of the arachnoid and close spatial relationship between the tumor surface and adjacent brain parenchyma.

Based on the above facts, it is thought that VEGF is synthesized in meningioma tumor cells. It is also thought that, through disruptions in the arachnoid, edema-inducing substances and macromolecules enter the peritumoral normal brain tissue and promote vascular permeability, giving impetus to edemagenesis in meningiomas.

Additional questions must be answered to support this hypothesis: (1) Do VEGF proteins exist in peritumoral brain tissues, and does peritumoral VEGF have a relationship with EI? (2) If so, it must be determined whether VEGF is produced by peritumoral brain tissues induced by ischemia and hypoxia. The present study examined the protein and mRNA expression of VEGF in human meningioma and peritumoral brain areas, which helps to answer these questions.

Our study shows that protein and gene expressions are congruent in the meningiomas and that both have a significant correlation with EI. In peritumoral areas, western blot results showed correlation with EI. However, mRNA was almost undetectable. In VEGF-positive cases, a decreasing gradient of VEGF expression was observed with increasing distance from tumors. This may explain why 28 meningiomas showed VEGF expression but only 21 had peritumoral edema. We found that VEGF mRNA could only be detected in tumors.

Moreover, VEGF has two major endothelial cell-specific, high-affinity receptors, flt-1 [14,31] and KDR [31,43]. Cerebral vessels of adults do not express VEGF receptors [45]. Further studies are needed to evaluate the expression of flt-1 and KDR in peritumoral brain tissues. If studies confirm these results, treatment with anti-VEGF antibodies could influence the extent of peritumoral brain edema. Other molecules, such as aquaporin-4 [48], may cooperate with VEGF in the genesis of brain edema. This should be tested by further research.
In conclusion, the present study demonstrates that peritumoral tissue does not produce VEGF and that the VEGF protein intensity in peritumoral tissues has a high correlation with EI. Thus, we conclude that VEGF macromolecules are secreted by tumor tissue and enter normal peritumoral tissue to induce edemagenesis in meningiomas.

Acknowledgements

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