VASCULAR ENDOTHELIAL GROWTH FACTOR AND BASIC FIBROBLAST GROWTH FACTOR EXPRESSION POSITIVELY CORRELATES WITH ANGIOGENESIS AND PERITUMOURAL BRAIN OEDEMA IN ASTROCYTOMA

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Background: Astrocytoma is the most malignant intracranial neoplasm and is characterized by high neovascularization and peritumoural brain oedema. Angiogenesis is a complicated process in oncogenesis regulated by the balance between angiogenic and antiangiogenic factors. **Methods:** The expression of two angiogenic growth factors, vascular endothelial growth factor and basic fibroblast growth factor were investigated using immunohistochemistry for astrocytoma from 82 patients and 11 normal human tissues. **Results:** The expression of vascular endothelial growth factor and basic fibroblast growth factor positively correlate with the pathological grade of astrocytoma, microvessel density numbers and brain oedema, which may be responsible for the increased tumour neovascularization and peritumoural brain oedema. **Conclusion:** The results support the idea that inhibiting vascular endothelial growth factor and basic fibroblast growth factor are useful for the treatment of human astrocytoma and to improve patient's clinical outcomes and prognosis.

Keywords: Angiogenesis, Peritumoural brain oedema, Vascular endothelial growth factor, Basic fibroblast growth factor.

INTRODUCTION

Astrocytoma is the most common intracranial neoplasms and can be divided into several grades according to its malignancy. However, the aetiology and pathophysiology are unclear and effective therapy is unavailable. Recently, tumour angiogenesis was found to be very important to tumour growth, invasiveness, metastasis and prognosis. Microvascular endothelial cells, a major component of microvasculatures exert angiogenic function via increased cell adhesion, migration, and capillary network formation.[1] Clinical studies found that density of microvessels is closely related to tumour malignancy and prognosis.[2] Therefore, elucidation of the pathophysiology of tumour angiogenesis will be helpful for developing therapeutic strategies for primary astrocytoma. Neovascularization and peritumoural brain oedema (PTBE) are the characteristic findings of the astrocytomas. The endothelial cell proliferation is forty times greater in high-grade gliomas than that in normal brain tissue.[3] In the avascular phase, the solid tumour are nourished through diffusion from their hosts' vasculature and can hardly grow beyond 2 mm in diameter whereas; in tumour neovasculature development and growth, the tumour vessels are characterized by increased vessel diameter, length, density and permeability.[4] The increased tumour vessels and angiogenic factors in astrocytoma contribute to peritumoural brain oedema[5,6], while in patients with glioblastoma the brain oedema is substantially alleviated by vascular normalization. The patients with gliomas accompanied by severe brain oedema often experiences poor clinical outcomes and the evaluation of the brain oedema by magnetic resonance imaging (MRI) is an independent prognostic factor in patients with malignant gliomas.[7]

Growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been well characterized in tumour angiogenesis.[8,9] In tumour angiogenesis, bFGF and VEGF are secreted by the tumour cells, as well as by platelets and potentially vascular mesenchymal cells.[10] These factors bind specific receptors on endothelial cells, which lead to the activation of the endothelial cell. Evidence has demonstrated that elevated bFGF and VEGF production by cancer cells directly correlates with tumour angiogenesis and tumour development.[11]

MATERIALS AND METHODS

Specimens from 82 patients with brain astrocytoma including 48 males and 34 females, ranging in age from 13 to 78 year-old (average, 37.3-year old) were collected during surgery from January 2004 to August 2006, in the First Affiliated Hospital of Medical College of XJTU. All brain specimens were obtained perioperatively from supratentorial surgical resection that rounded the rim of tumours, and divided into four groups according to the new World Health Organization (WHO) classification of brain tumours.[12] Grade I, 16 cases of pilocytic astrocytoma; Grade II, 22 cases, included 15 cases of pilomyxoid astrocytoma and 7 cases of pleomorphic xanthoastrocytoma; Grade III, 24 cases of anaplastic astrocytoma; Grade IV, 20 cases, included 16 cases of glioblastoma and 4 cases of gliosarcoma. Eleven normal brain specimens including cortex and white matter were obtained from autopsy cases without any evidence of brain tumour or other brain diseases, which included six cases of male and

five cases of female ranging in age from 13 to 62 years (Mean, 38.2 years). All persons gave their informed consent prior to their inclusion in the study. Within 10 min after surgical resection, these tissues were fixed with freshly prepared 10% formalin at 4 °C for 24 hour and embed in paraffin.

Formalin fixed, paraffin-embedded tissues were serial sectioned at 5μm thick and collected onto slides which had been coated with Poly-L-lysine. Staining was performed using immunoperoxidase-staining kits for mouse and rabbit both with goat immunoglobulin. After routine deparaffinization and rehydration, tissue sections were incubated with 3.0% hydrogen peroxide at room temperature (RT) for 10 min in order to quench the endogenous peroxidase activity. The slides were placed in a glass jar filled with 10 mM sodium citrate buffer (*p*H 6.0) and were boiled for 10 min within a microwave oven to retrieve the antigens. Slides were cooled down at RT for 20 min and then washed with phosphate buffered saline (PBS). Sections were then incubated with 5% bovine serum albumin (BSA) at RT for 20 min to block non-specific antigen. The sections were incubated with primary antibodies as follows: CD105 (Endoglin) mouse monoclonal antibody (NCL-CD105, Novocastra, UK, 1:100 dilution in PBS, overnight at 4 °C); VEGF (C-1) mouse monoclonal antibody (sc-7269, Santa Cruz Biotechnology Inc., USA, 1:600 dilution in PBS, overnight at 4 °C); bFGF (147) rabbit polyclonal antibody (sc-79, Santa Cruz Biotechnology Inc., USA, 1:800 dilution in PBS, overnight at 4 °C). After rinsed with PBS for three times, the sections were incubated with biotinated goat anti-mouse immunoglobin (for VEGF and CD105), or biotinated goat anti-rabbit immunoglobin (for bFGF) at 37 °C for 20 min. Then sections were rinsed and incubated with avidinbiotin-horseradish peroxidase complex at 37 °C for 20 min. The sections were visualized with 3,3-diaminobenzidine tetrahydro-chloride and counterstained with hematoxylin. Specimens were dehydrated and mounted for an observation under microscope. The primary antibodies were substituted with PBS and 1.0% BSA as a negative control for each section.

Two pathologists who had no knowledge of the patients' pathological diagnosis and clinical data observed the staining results of immunohistochemistry. One thousand cells in five random high-powered fields at ×400 magnification were observed per slide. The immuno-positive cells for VEGF and bFGF were counted and the positive rate was described as labelling index (LI).

With the CD105 staining, the MVD of each section was calculated according to Wendner's criteria.[13] Briefly, each section was observed under low-powered fields (LPF) at ×100 magnification to find the 'hot spots'. Then the microvessels of each 'hot spot' were counted at ×200 magnification field. A single endothelial cell or cluster of endothelial cells, which were stained dark brown and clearly separated from adjacent astrocytoma cells or connective tissues, were recognized as a countable microvessel. Five randomly selected fields at ×200 magnification per slide were evaluated, and the mean MVD was calculated.

The volume of astrocytoma and PTBE of each case was evaluated by magnetic resonance images (MRI).[14] The volume of tumour (V_{tumour}) was assessed by gadoliniumdiethylenetriamine pentaacetic acid (Gd-DTPA)-enhanced T1-weighted images, and the volume of PTBE (V_{oedema}) was evaluated by T2-weighted images or fluid-attenuated inversion-recovery (FLAIR) images. In each case, we measured the maximum coronal (a), axial (b), and sagittal (c) diameters, which were perpendicular to each other. The volumes of tumour and PTBE were calculated according to the formula: $V=4/3\pi(a)(b)(c)$. The relation between the volume of tumour and PTBE in each case was defined as oedema index (EI): $EI=(V_{tumour}+V_{oedema})/V_{tumour}$.

The data are presented as the mean ± standard error. One-way-ANOVA was used for statistical comparisons of the LI, MVD and EI between each grade of astrocytoma. Statistical significance was defined as $p<0.05$. The correlations between MVD, EI and LI VEGF and bFGF were analyzed by Spearman's rank correlation.

RESULTS

MVD in astrocytoma and normal brain tissue

Endothelial cells labelled by CD105 were detected more in astrocytoma than in normal brain tissue. The microvessels in astrocytoma were in lumen-, sprout-, and glomerulus-like pattern. There were statistical differences in MVD between normal brain tissues vs. WHO grade I-IV astrocytoma. The MVD was increased with the pathological grade of astrocytoma and was higher in grade I vs. grade III and IV; grade II vs. grade IV ($p<0.05$). The MVD number positively correlate with the pathological grade of astrocytoma (Spearman's rank correlation, $r=0.608$, $p=0.01$. Table-1, Figure-1 A-C and D.

Table-1: The MVD number and the LI of VEGF and bFGF in normal brain tissues and different grades of astrocytoma (Mean±SE)

Grades	Normal	Grade I	Grade II	Grade III	Grade IV
MVD Number	1.61±0.20	9.90±1.06	13.25±1.52	19.67±1.93	23.83±1.31
VEGF LI (%)	5.12±0.78	25.39±3.31	38.79±0.99	41.91±1.27	49.33±1.55
bFGF LI (%)	14.75±0.94	33.43±1.15	38.54±1.45	43.70±1.22	49.54±1.58

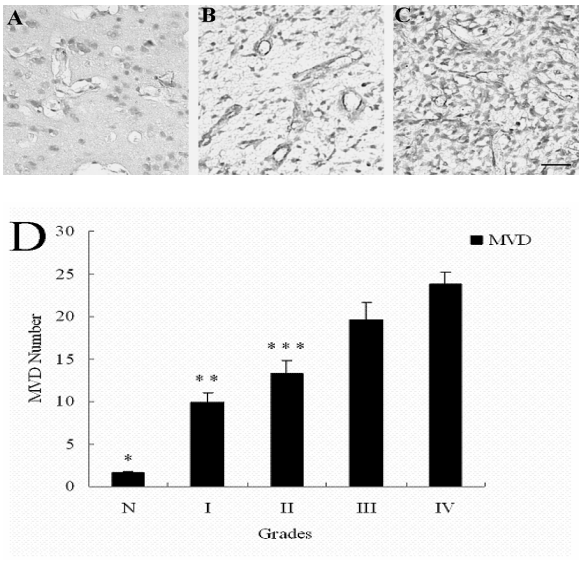

Figure-1: A-C and D. The MVD number in normal brain tissues and different grades of astrocytoma. The MVD number in normal brain tissues (A) is lower than low-grade astrocytoma (B), and higher in high-grade astrocytoma (C). Scale bar=100 µm. The MVD number is increased with the elevated grade of astrocytoma (Spearman's rank correlation, r=0.608, p=0.01) (D).

*Significantly different (p<0.05) vs. Grade I-IV astrocytoma.
**Significantly different (p<0.05) vs. Grade III and Grade IV astrocytoma.
***Significantly different (p<0.05) vs. Grade IV astrocytoma

VEGF and bFGF expression in astrcytoma and normal brain tissue

Positive immunostaining for VEGF was located in the cytoplasm of endothelial cells, astrocytoma cells, and small part of astrocytic cells. Normal brain tissues seldom expressed VEGF, while mild expression was found in low-grade astrocytoma and higher expression in high-grade astrocytoma. The VEGF LI in normal brain tissue was significantly different from that of grade I-IV glioma, also in grade I vs. grade II, III and grade IV and grade II-III vs. grade IV (p<0.05). The VEGF LI increased with the rising grade of astrocytoma and positively correlated with the grade of astrocytoma (Spearman's rank correlation, r=0.772, p=0.01). Positive staining for bFGF was found in the cytoplasm of astrocytoma cells, endothelial cells, and astrocytic cells. The bFGF expression was higher in high-grade astrocytoma than in low-grade ones and lower in normal brain tissues. The bFGF LIs of the five groups were significantly different from each other (p<0.05). The bFGF LI was positively correlated with tumour grades (Spearman's rank correlation, r=0.808, p=0.01). (Table-1, Figure-2 D-I and J).

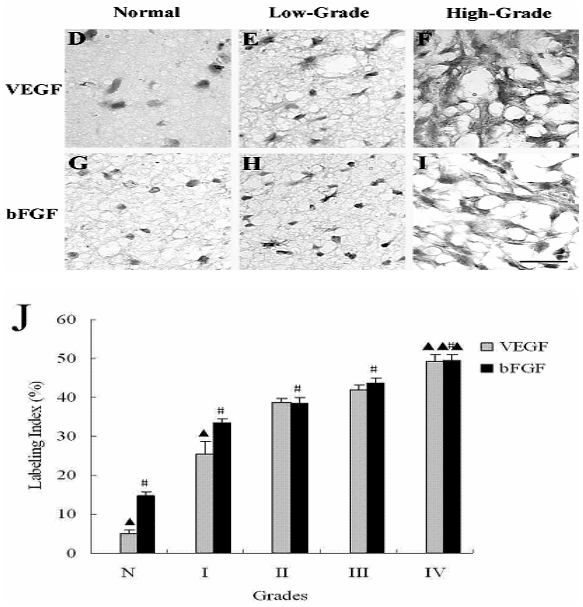

Figure-2: (D-I and J). The LI of VEGF and bFGF in normal brain tissues and different grades of astrocytoma. The expression of VEGF and bFGF are higher in high-grade astrocytoma (F, I) than low-grade astrocytoma (E, H), and lower in normal brain tissues (D, G). Scale bar=25 µm. The LI of VEGF and bFGF is increased with the elevated grade of astrocytoma (VEGF: Spearman's rank correlation, r=0.772, p=0.01; bFGF: Spearman's rank correlation, r=0.808, p=0.01).

VEGF: ▲Significantly different (p<0.05) vs. Grade I-IV astrocytoma; ▲▲Significantly different (p<0.05) vs. Grade II, III and IV astrocytoma; ▲▲▲Significantly different (p<0.05) vs. Grade II and III astrocytoma. *bFGF*: #Significantly different (p<0.05) in all Grades (J)

Correlation between MVD and LI of VEGF/bFGF

The VEGF LI and bFGF LI increased, with the increasing tumour grade and MVD. Therefore, we investigated the correlation between MVD and the LI of VEGF and bFGF. All specimens were divided into four groups according to MVD numbers: Group A: 1–10; Group B: 10–20; Group C: 20–30; Group D: 30 or more. There was significant difference of VEGF expression in Group A vs. Group B, Group C, and Group D, also Group B vs. Group D (p<0.05); and there was positive correlation between MVD and VEGF LI (Spearman's rank correlation, r=0.522, p=0.01). The bFGF expression in Group A was significantly different from Group B, Group C, and Group D (p<0.05); and positively correlated with MVD (Spearman's rank correlation, r=0.633, p=0.01). (Table-2, Figure-3).

Table-2: The LI of VEGF and bFGF in specimens of different MVD grades (Mean±SE)

MVD Grades	Group A	Group B	Group C	Group D
VEGF LI (%)	23.28±2.60	36.97±2.33	42.18±1.90	51.40±2.24
bFGF LI (%)	27.60±1.73	40.57±1.43	45.95±1.58	47.29±3.02

EI positive correlation with MVD number and VEGF, bFGF expression

The MVD number was seriously influenced by the quantity of neovascular, so the correlation between MVD

and EI was further investigated. We divided astrocytoma specimens into four groups according EI: Group 1, EI ranged from 1 to 2; Group 2, from 2 to 4; Group 3, from 4 to 6; Group 4, above 6. When we focused on MVD number, significant differences were found between Group 1 vs. Group 2, Group 3 and Group 4, and also between Group 2 vs. Group 3 and Group 4 (p<0.05). The MVD number increased with the increasing EI, and was positively correlated with EI (Spearman's rank correlation, r=0.533, p=0.01). Our study had shown that EI was positively correlated with MVD number. (Table-3, Figure-4).

Furthermore, we analyzed the correlation between EI and expression of VEGF and bFGF. The VEGF LI of group 1 was significantly lower than that of Group 3 and Group 4, also Group 2 was lower to Group 4 (p<0.05). PTBE was aggravated by the increased in the expression of VEGF. VEGF LI was positively correlated with EI (Spearman's rank correlation, r=0.500, p=0.01). Statistical differences of the bFGF LI were found between Group 1 vs. Group 2, Group 3 and Group 4; also between Group 2 vs. Group 3 and Group 4 (p<0.05). The expression of bFGF was positively correlated with EI (Spearman's rank correlation, r=0.589, p=0.01). (Table-3, Figure-5).

Table-3: The MVD number and the LI of VEGF and bFGF in specimens of different EI grades (Mean±SE)

EI Grades	Group 1	Group 2	Group 3	Group 4
MVD Number	10.33±1.16	15.18±1.78	22.18±2.10	20.76±1.58
VEGF LI (%)	30.47±2.85	37.33±2.04	42.98±2.28	46.49±1.32
bFGF LI (%)	34.79±1.37	39.13±1.22	44.67±1.81	48.03±1.56

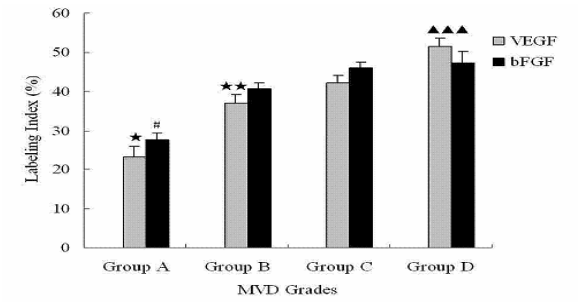

Figure-3: The LI of VEGF and bFGF in specimens of different MVD grades. The LI of VEGF (Spearman's rank correlation, r=0.522, p=0.01) and bFGF (Spearman's rank correlation, r=0.633, p=0.01) is increased when the grade of MVD is elevated.

VEGF LI: ▲Significantly different (p<0.05) vs. Group B, Group C and Group D; ▲▲Significantly different (p<0.05) vs. Group D. *bFGF LI*: #Significantly different (p<0.05) vs. Group B, Group C and Group D.

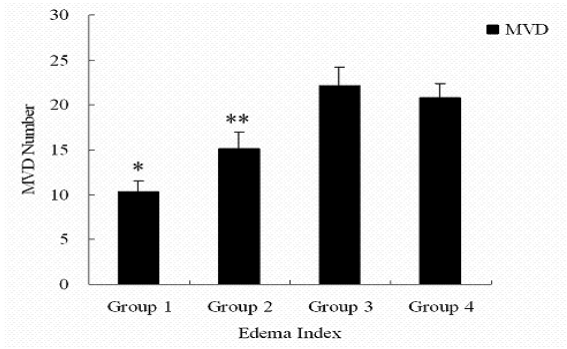

Figure-4: The MVD number in specimens of different EI grades. The MVD number is increased when the grade of EI is elevated (Spearman's rank correlation, r=0.533, p=0.01).

*Significantly different (p<0.05) vs. Group 2, Group 3 and Group 4. **Significantly different (p<0.05) vs. Group 3 and Group 4.

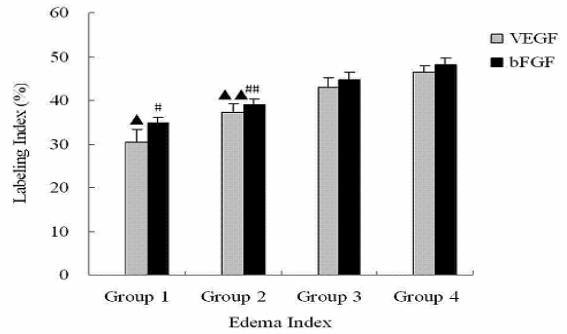

Figure-5: The LI of VEGF and bFGF in specimens of different EI grades. The LI of VEGF (Spearman's rank correlation, r=0.500, p=0.01) and bFGF (Spearman's rank correlation, r=0.589, p=0.01) is increased when the grade of EI is elevated.

VEGF LI: ▲Significantly different (p<0.05) vs. Group 3 and Group 4. ▲▲Significantly different (p<0.05) vs. Group 4. *bFGF LI*: #Significantly different (p<0.05) vs. Group 2, Group 3 and Group 4. ## Significantly different (p<0.05) vs. Group 3 and Group 4.

DISCUSSION

The growth of tumours relies on the formation of their own vascular network, which provides nutrition and oxygen to the tumours, and removes carbon dioxide as well as metabolic waste products. This study investigated that angiogenesis factors modulate the neovascularization of astrocytoma and influence PTBE. Tumour neovascularization promotes the neoplasms growth and progression. We analyzed the expression of two potent angiogenic factors, VEGF and bFGF. In our study, VEGF strongly expressed in high-grade astrocytoma (grade III and grade IV) cells, mildly expressed in low-grade, and seldom expressed in normal brain tissues. VEGF LI was positively correlated with elevated pathological grade of astrocytoma and MVD numbers. VEGF acted as a

mitogenic factor specifically on endothelial cells, through which it promotes neovascularization in tumour progression.[15] VEGF expresses in a variety of human malignant tumours, such as gastric cancer, breast cancer, and lung cancer as well, and is closely correlated with intratumour angiogenesis and poor prognosis.[16–18] VEGF over-expression in astrocytoma significantly correlated with tumour pathological grades, proliferation and malignant transformation.[19] Another potent angiogenesis factor is bFGF, which is over expressed in breast cancer, hepatocellular cancer, and lung cancer as well.[20–22] Our results showed that bFGF expression was increased with elevated astrocytoma grades and MVD, and was positively correlated with tumour grades as well as MVD. VEGF and bFGF might promote tumour progression by inducing neovacularization as well as acting directly on tumour cells.[23]

CONCLUSION

Our study concluded that increasing VEGF expression was correlated with severe PTBE in astrocytoma, which indicated that VEGF contributed to the formation of peritumoural brain oedema. Present results also demonstrate that high MVD correlates with severe peritumoural oedema in patients with astrocytoma. It is also concluded that bFGF expression was positively correlated with PTBE and MVD numbers, which indicated that bFGF, aggravate the PTBE in patients with astrocytoma through inducing tumour neovascularization.

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