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Expression of bFGF and VEGF in Brain Astrocytoma

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Neovascularization is an important factor in the prognosis of brain tumor and many angiogenetic factors have been evaluated for prognostic significance. Among them, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are known as potent angiogentic factors and mitogens. We evaluated seven cases of grade II brain astrocytoma. Four, group A, was diagnosed as anaplastic progression at their second operation, and three, group B, did not. Using monoclonal antibodies to bFGF and VEGF in paraffin embedded tissue from first operation, their immunoreactivity and differences between two groups were examined. The growth fractions of these tumor were also measured by Ki-67 monoclonal antibodies (MIB1). Immunostaining for bFGF in tumor cells were observed in both nuclei and cytoplasm, and for VEGF, mainly observed in the cytoplasm. Mean cell count number ± standard deviation per high power field in each were as follows : 1) for bFGF, 20.08 ± 6.38 in group A and 0.87 ± 0.90 in group B ($P < 0.01$), 2) for VEGF, 43.75 ± 17.09 in group A, and 0.8 ± 1.06 in group B ($P < 0.05$) and 3) for the proliferation index with Ki-67 antibodies, 3.20 ± 0.81 in group A and 0.77 ± 1.03 in group B ($P < 0.05$). This data supports the assertion that angiogenetic factor such as bFGF and VEGF may contribute to progressive change of astrocytoma by tumor angiogenesis.

Key Words : *bFGF, VEGF, Brain tumor*

INTRODUCTION

Neovascularization is important in tumor growth, invasiveness, vascular permeability, and edema, and associated with malignant progression (Brem et al., 1978). The growth of blood vessels may be a crucial step in the growth of human and experimental brain tumors (Heuser and Miller, 1986). Determining events leading to angiogenesis include 1) release of angiogenic factors (AFs), 2) secretion of proteases that release AFs stored in the extracellular matrix, 3) chemotaxis for macrophages which subsequently release AFs, and 4) release of endothelial cells from inhibitory control. AFs stimulate *in vivo* neovascularization and are called "direct" when they stimulate endothelial cell division or migration *in vivo* and *in vitro* (Zagzag, 1995). "Indirect" AFs have no direct mitogenic effect on endothelial cells *in vitro* but are able to promote angiogenesis *in vivo*, probably in part by stimulation of target cells to release direct AFs (Folkman and Klagsbrun, 1987).

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Growth factor currently appears to be the central mediator of angiogenesis. The fibroblast growth factor (FGF) family is composed of at least nine related mitogens that affect a varity of cells of neuroectodermal or mesenchymal origin. FGF-1, or acidic FGF, and FGF-2 or basic FGF, share 53 % sequence homolgy (Zagzag, 1995). Basic FGF (bFGF) is more effective than acidic FGF (aFGF) (Folkman and Klagsbrun, 1987). They have a strong affinity for heparin and are often associated with the heparan sulfate proteoglycans (HSPGs) present in the basement membrane and extracellular matrix. bFGF, also described as a fibroblast mitogen and isolated from bovine pituitary glands and brains, is known to be synthesized by a varity of tumor and endothelial cells (Basilico and Moscatelli, 1992). It is found in reactive astrocytes (Finkelstein et al., 1988) and neurons (Janet et al., 1988). Since it has been known as it is a potent mitogen for astroglial cells (Weibel et al., 1985), this factor is also known to be capable of stimulating the replication of multiple cell types including astrocytes (Kniss and Burney, 1988) and glioma cells (Morrison et al., 1990). bFGF is a direct AF which promotes every phase of the angiogenetic process and it induces *in vitro* synthesis of plasminogen activator, a serine protease that plays a critical role in the angiogenetic process (Moscatelli and Rifkin, 1988) and other proteases.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a dimeric glycoprotein with 36,000 to 46,000 molecular weight and has been identified from the conditioned medium of several cell lines (Favard et al., 1991). VEGF 1) increases blood vessel permeability, 2) stimulats endothelial cell division *in vitro*, and 3) induces angiogenesis *in vivo* (Pötgens et al., 1995). VEFG is about 1,000-fold more potent than histamine in inducing capillary permeability. Apart from its expression by some normal, well vascularized, and embryonic tissues, and during wound repair, VEGF may play a role in the development and maintenance of normal and tumor-associated vasculature (Weindel, 1994).

Of the four different isoforms arising from alternative mRNA splicing ($VEGF_{121,165,189,206}$), $VEGF_{165}$ is the most abundant and best characterized form (Zagzag, 1995). Colocalization of [^{125}I] VEGF binding with factor VIII-like immunoreactivity proved the existence of VEGF binding sites on vascular endothelial cells (Jakeman et al., 1992). Two different receptors for VEGF have been characterized : fms-like tyrosine kinase (flt) and fetal liver kinase1 (flk-1/kinase insert domain-containing receptor (KDR). Both receptors are expressed in human microvessel and large-vessel endothelial cells.

Abundant neovascularization in a hallmark of malignant glioma. But, there were few studies on the possible role of these angiogenetic factor such as bFGF and VEGF, in human astrocytoma (Stefanik et al., 1991). Evidences available for bFGF involvement in the pathogenesis of human gliomas are ; 1) proliferative effect on cultured glioma cells (Westphal et al., 1988) and expression of the aFGF gene and FGF receptors in glioma cell lines (Libermann et al., 1987), and 2) that bFGF is found in reactive astrocytes (Finkelstein et al., 1988) and neurons (Janet et al., 1988).

This study's object was to evaluate the expression of bFGF and VEGF in brain astrocytoma by histopathologic examination and immunohistochemistry. We tried to determine whether the level of bFGF and VEGF expression correlated with the malignant progression of brain astrocytoma.

MATERIALS AND METHODS

Patients and tumor histology

Data for this study were collected from patients with brain astrocytoma, who had undergone brain tumor surgery at least twice at the KangNam Sacred Heart hostpital from 1988 to 1995 ; there were seven patients. Sugical specimens were fixed in 10 % buffered formalin and paraffin embedded. Routine sections stained with hematoxylin and eosin (H&E) were reviewed and histologic grading was re-performed. In four patients, there was progression of tumor grade in the second biopsy (group A), and in three patients, no change in tumor grade (group B). The distribution of clinical and pathologic data for the entire patient population is listed in Table 1.

Immunohistochemistry

Staining for immunohistochemistry was performed using a commonly used method. Briefly, formalin-fixed, paraffin-embedded 5 μm sections were rehydrated and incubated overnight at 4℃ with the primary antibody diluted. Biotinylated antimouse IgG (1 : 100, Signet Laboratories Inc, MA) and a complex of streptavidin biotinylated peroxidase (1 : 100, Signet

Table 1. Summary of immunohistochemical results

	Sex/Age	Site	MIB1(%)	bFGF	VEGF
A	F/47	Rt. temporal	2.25	24.52	26.8
	M/38	Lt. frontal	3.17	11.4	34.8
	M/23	cerebellum	4.23	19.2	47.4
	M/28	Lt. frontal	3.15	25.2	66
	mean±SD		3.20±0.81	20.08±6.38	43.75±17.09
B	F/17	Rt. temporal	0.31	1.8	0.4
	M/38	Lt. frontotemporal	1.95	0.8	0
	F/33	Rt. frontal	1.06	0	0.8
	mean±SD		0.77±1.03	0.87±0.90	0.8±1.06
	P value		P=0.03	P=0.01	P=0.02

SD : standard deviation

Laboratories Inc, MA) were added in sequence, followed by 3-amino 9-ethylcarbazole (AEC) in organic solvent and the slides were counterstained with Mayer's hematoxylin.

1) bFGF

A mouse monoclonal antibody to recombinant bFGF (Oncogene science, MA) was used for immunohistochemical stain. An adenocarcinoma specimen of the colon was used as positive control and sections with ommision of primary antibody were used as a negative control.

2) VEGF

A rabbit polyclonal antibody generated by immunizing rabbits with a peptide from the N-terminus region of $VEGF_{165}$ (Oncogene science, MA) was used. Fetal tissue was used for positive control and normal adult brain tissue from autopsy specimen was used as a negative control.

3) Ki-67

Mouse monoclonal MIB1 antibody to the Ki-67 nuclear antigen (Immunotech S.A., France) was used. Sections of these tumors were incubated with the Ki-67 monoclonal antibody to determine the growth fraction of the tumor.

4) Evaluation

The cells with positive reaction were counted in five X 400 microscopic fields (Olympus, BH2 microscope, about 0.19 mm^2 per field with the field size measured with an ocular micrometer) in areas with most active neovascularization and the average numbers of stained cells were calculated.

Results are expressed as the mean ± standard deviation. The *t*-test was used to determine significant differences between A and B groups. Differences with P values less than 0.05 were considered statistically significant. These evaluations were done by two observers completely blinded to the informations about patients.

RESULTS

H & E staining

Hematoxylin and eosin staining of seven cases of grade II out of four grades brain astrocytoma revealed slight hypercellularity, mild to moderate nuclear

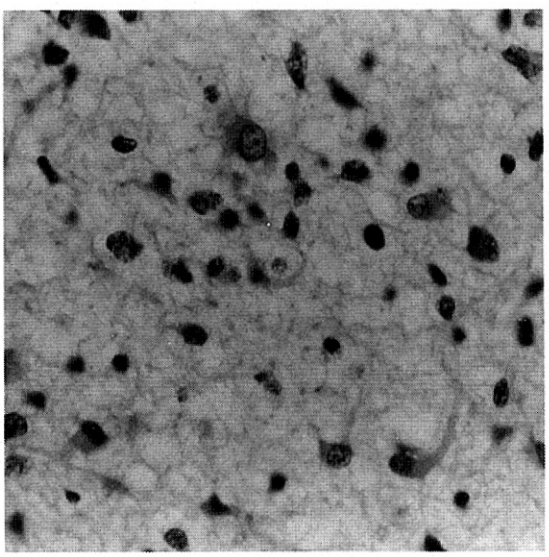

Fig. 1. Grade II brain astrocytoma of group A reveals slight hypercellularity, moderate nuclear pleomorphism, and few mitoses(H&E).

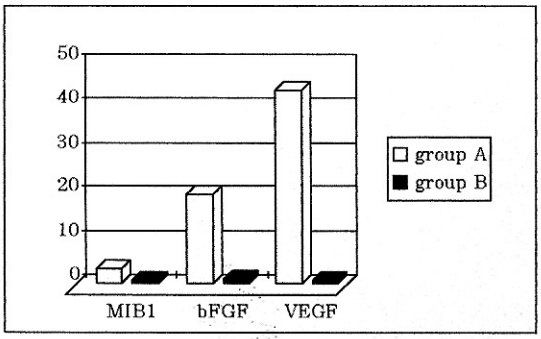

Fig. 2. Distribution of immunohistological expression of MIB1, bFGF, and VEGF. (The number on the left represents percentage for MIB1, and mean count number of positive stained cells per high power field for bFGF and VEGF)

pleomorphism, hyperchromatic nuclei, and few mitotic figures(Fig. 1). Areas of necorsis with or without pseudopalisading were not seen in any cases. Hyperplastic blood vessels revealed a mild degree of endothelial proliferation.

bFGF

In normal brain tissue, bFGF could be seen focally in the perivascular matrix of large blood vessels.

The results of bFGF immunostaining in tumor are summarized in Table 1 and schematically presented in Fig. 2. Positive immunostaining for bFGF was observed in six out of seven cases(85.7 %). Mean count number of positively stained tumor cell per high power field was 20.08 in group A and 0.87 in group B ($P < 0.01$), and the immunoreactivity was more prominent in group A than group B. In tumor vessels, the nuclei of endothelial cells showed positive reaction (Fig. 3a). The positive reaction of endothelial cells were found in a) hyperplastic vessels in the main tumor masses, b) the infiltrative invasive zone of the tumor, and c) variably brain adjacent to the tumor. Vessels distant from the tumors were negative. Reactive astrocytes seen in the brain adjacent to the tumor variably showed positive reaction.

In the tumor cell compartment, fibrillary astrocytes with positive immunoreaction were usually diffusely distributed. The tumor cells showed diffuse positive reaction in nuclei (Fig. 3b). Tumor cell cytoplasm showed focal positive reaction, which was heterogenous and relatively rare (Fig. 3c). Generally, the large tumor cells expressed bFGF, compared to negative reaction in the small cells with scanty cytoplasm.

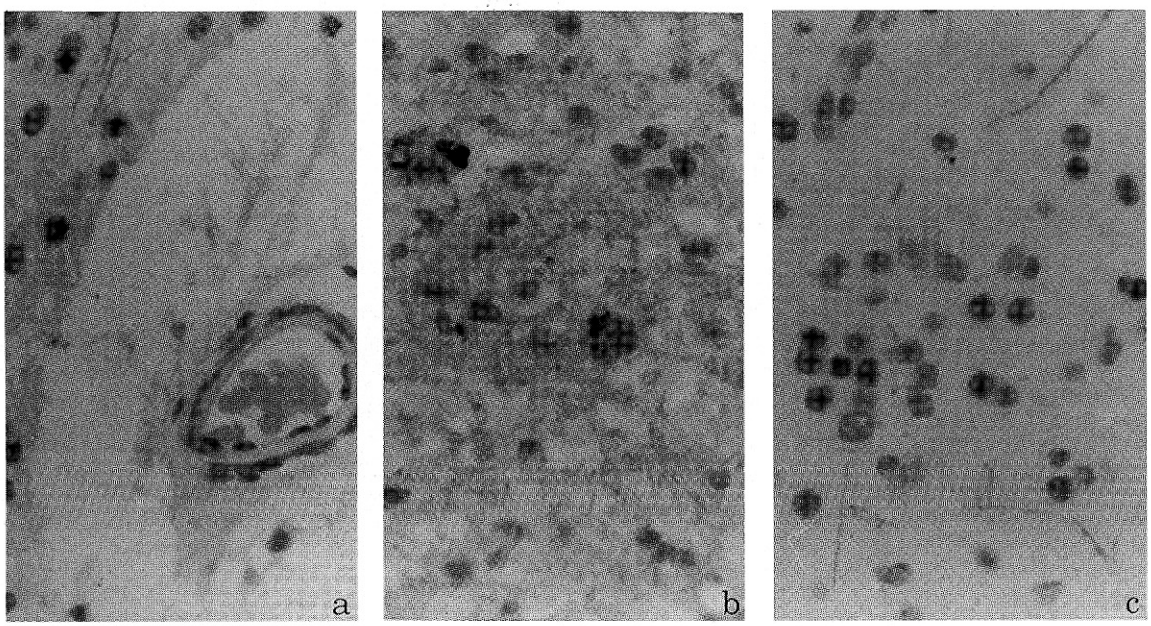

Fig. 3. bFGF immunoreactivity in grade II astrocytoma of group A : diffuse positive reactions in nuclei of endothelial cells of tumor vessels(a) and nuclei of tumor cells(b), and a heterogenous and relatively rare focal positive reaction in tumor cell cytoplasm(c).

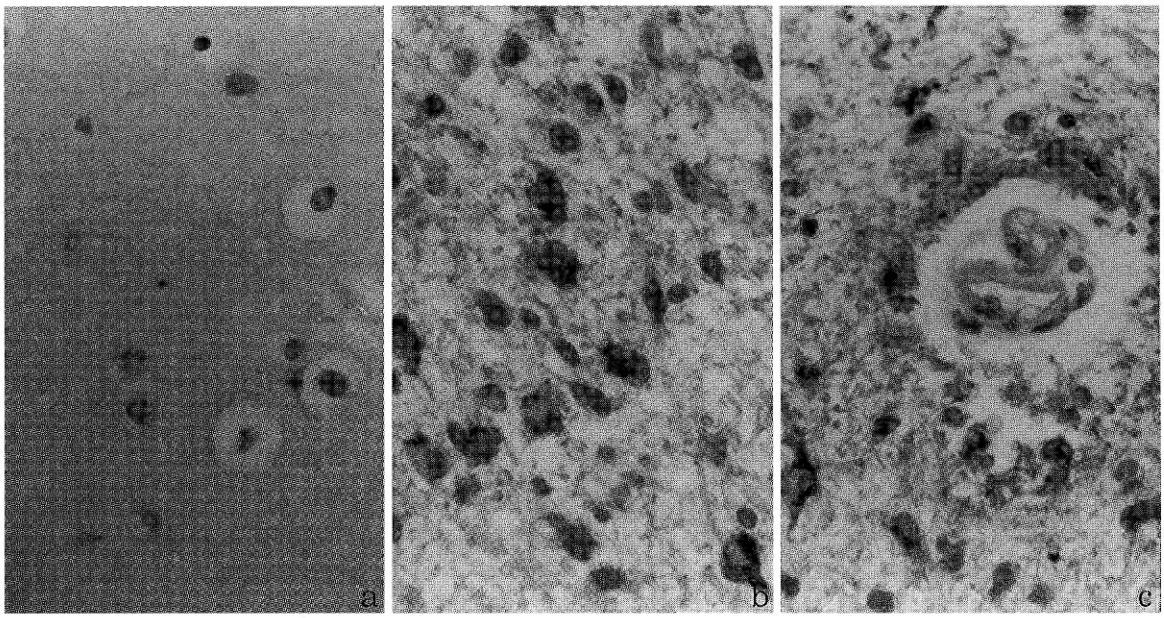

Fig. 4. VEGF immunoreactivity : negative in normal brain(a) and strong positive reactions in perivascular matrix(b) and cytoplasm of tumor cells(c) in astrocytoma of group A.

VEGF

Normal adult brain showed negative reaction to VEGF(Fig 4a).

The results of the VEGF immunostaining are summarized in Table 1 and schematically presented in Fig. 1. Positive immunostaining for VEGF was observed in six out of seven cases (85.7 %). Mean cell count number of positively stained tumor cell per high power field was 43.75 in group A compared with 0.8 in group B (P 0.05). The immunoreactivity was more prominent in group A than group B. In the tumor portion, strong VEGF positive staining was seen in the perivascular matrix of tumor vessels (Fig. 4b). In the tumor cell compartments, the expression of VEGF was mainly identified in the cytoplasm (Fig. 4c) of tumor cells along capillaries and clusters of tumor cells.

Ki-67 index(MIB1)

As determined by Ki-67 labeling, group A showed more high growth fraction (3.20 %) than group B (0.77 %) ($P < 0.03$) (Table 1 and Figs. 1 and 5).

DISCUSSION

Neovascularization is a neccessary phenomenon for both normal and neoplastic growth. Tumor growth

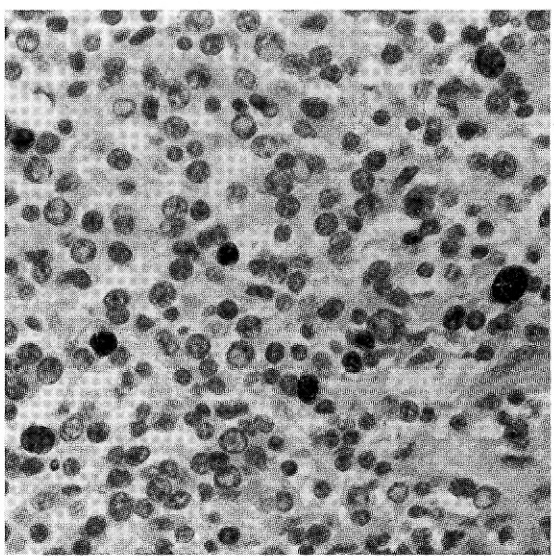

Fig. 5. MIB1 was stained in the nuclei of tumor cells(group A).

is dependent upon the ability of the tumor to recruit a vasculature. Glioblastoma multiforme (GBM) is a high grade lethal brain tumor characterized by aberrant neovascularization with thick walled proliferative vascular channels and tendency to regrow regardless of the treatment administered (Weindel et al., 1994). Low grade gliomas may during the course of time undergo anapalstic development and become dedifferentiated fast-growing neoplasms during the course of time. This progressive change is characterized by high cellular density, nuclear and cellular pleomorphism, mitosis, the presence of necrosis, capillary sprouting, and endothelial cell proliferation (Daumas-Duport et al., 1988). At the present time the fundamental mechanism that triggers the cascade of events that lead to the progression of an initially low-grade quiescent or slow-growing glioma to a highly proliferative anaplastic tumor with a dense three-dimensional capillary network is not fully known.

The bFGF has been demonstrated at first in bovine corneal basement membrane and in developing retinal capillaries (Hanneken, 1989). Endothelial cells synthesize and store bFGF within the cell and its extracellular matrix, which *in vivo* includes the basement membrane of the microvascular wall. Folkman et al. hypothesized that tissue injury could release bFGF, thererby triggering angiogenesis (Folkman et al., 1988). They may also serve as reservoirs for the vascular mitogens. The bFGF exists as either a stable, inactive molecule or a rapidly degradable, bioactive molecule (Bashkin et al., 1989). The active form of bFGF stimulates endothelial cell mitosis, migration, and remodeling of basement membranes, which are three major steps in the process of angiogenesis (Ausprunk and Folkman, 1977). The microvessels in situ in malingnant brain tumors contain bFGF, a potent cellular mitogen capable of stimulating both angiogenesis and tumorigenesis (Brem et al., 1992). The matrix components in the basement membrane that bind to bFGF can also release the mitogen locally, which stimulates the adjacent tumor cells with bFGF receptors in a paracrine manner (Gross et al., 1992). With lacking a signal peptide, bFGF remains sequestered in a functionally inactive state (Vlodavsky et al., 1987), which explains the observation that the endothelium is mainly quiescent and nonproliferative, even in highly vascular brain tumors(Tsanaclis et al., 1991).

The presence of RNA coding acidic and basic FGFs in glioma was demonstrated by Takahashi et al. using Northern blot analysis (Takahashi et al., 1990). In their study, 17 of 18 gliomas expressed basic FGF, while 13 of 18 expressed acidic FGF. Immunohistochemistry has confirmed the presence of acidic FGF, basic FGF, and receptors for basic FGF in cultured, malignant glial cells. Neither bFGF nor any other trophic factor acts alone in isolation from other cytokines (Sporn and Roberts, 1988); ultimately, the *in vivo* response of a regulatory peptide such as bFGF is the combined result of multiple regulatory growth factors working in concert. Additional angiogenic molecules, epidermal growth factor, transforming growth factor-α, platelet-derived growth factor, and renin have been described as mediators of neovascularization in human brain tumors (Shibura et al., 1988). A considerable difference was reported in the staining of malignant as opposed to normal brain tissue for both acidic and basic FGF (Stefanik et al., 1991). The bFGF was known to show positive reaction in the perivascular matrix. Gliobliostoma showed abundant positive reaction in the matrix surrounding proliferating blood vessels. In our study, basic FGF was mainly demonstrated in the nuclei of astrocytic tumor cells and in the matrix surrounding proliferating vessels. In Stefanik's study, it was reported that basic FGF was mainly demonstrated in the matrix surrounding proliferating vessels and showed inhomogeneous reaction in the tumor cell (Stefanik et al., 1991). But, it is of interest that Zagzag et al. found that bFGF expression was characteristic of malignant but not low grade astrocytomas (Zagzag et al., 1990); the capillary endothelium was immunoreactive within and at the margin of malignant astrocytomas but not in the brain distant to the tumor.

VEGF is known to be a specific growth factor for endothelial cells because its receptor is selectively present in endothelium. The potent mitogenic activity of VEGF for endothelial cells has been characterized and is considered to be comparable to that of bFGF, which has been known to be the most potent mitogen for endothelium among many angiogenic factors. VEGF possesses a signal peptide and it is largely free to diffuse in tissues after secretion compared to bFGF that lacks it. It is also known that VEGF plays a crucial role in vasculogenesis; therefore, expession of VEGF might directly contribute to the promotion of angiogenesis. VEGF is overexpressed in a number of processes that are characterized by a common set of pathophysiological features that include the following: hyperpermeable blood vessels with extravasation of

plasma proteins, clotting of extravasated plasma fibrinogen to fibrin, replacement (organization) of fibrin by well vascularized connective tissue stroma (granulation tissue), and remodeling of granulation tissue into hyalinized and relatively avascular connective tissue (referred to as desmoplasia, scar, corpus albicans in tumors, healed wounds, and cycling ovaries, respectively) (Kamat et al., 1995). In all of these examples, fibrin provides a provisional stromal matrix that induces and supports the ingrowth of new blood vessels as well as the fibroblasts responsible for synthesis and secretion of the structural proteins and proteoglycans that typically comprise mature connective tissue. VEGF is thought to have an important role in initiating these events, serving as a selective endothelial cell mitogen and rendering blood vessels hyperpermeable to plasma proteins, thereby provoking fibrinogen extravasation and consequent deposition of a fibrin provisional matrix.

VEGF gene, mRNA, and proteins are expressed by animal and human glioma cell lines (Plate et al., 1993) and VEGF is immunolocalized in astrocytomas (Alvarez et al., 1992). VEGF is known to be expressed in a variety of tumor cell types, especially in tumor tissues with rich vascularization such as glioblastomas, bladder tumor, and renal cell carcinomas. (Berkman et al., 1993). Immunoreactivity was detected around necrotic areas in the pseudopalisading cells of glioblastomas, in tumor cells along capillaries (Shwieki et al., 1992), and in clusters of tumor cells (Plate et al., 1992).

The relationship between the grade of brain tumor and the quantitative levels of VEGF has not been established yet. Weindel et al. (1994) reported that in spite of no obvious correlations between expression levels of two known VEGF receptors in brain tumor, gene expression of VEGF was significantly elevated in high grade gliomas compared to low grade tumors. Like basic FGF, VEGF seems to increase the secretion of proteolytic enzymes (Unemori et al., 1992), which was increased in high grade brain tumors (Gross et al., 1988). Although there was not enough evidence that expression of VEGF could be proposed as an explanation for the anaplastic progression of brain gliomas or as exclusive cause for capillary and endothelial proliferation in brain tumors, the concentration of VEGF activity measured in cyst fluid was elevated up to 45 fold in patients with high grade gliomas compared to low grade astrocytomas or human plasma. (Weindel et al., 1994). So, VEGF seemed rather to represent the key molecule for starting neovascularization and a sensitive measure of the angiogenic potential of a tumor. It is therefore not suprising that pilocytic astrocytomas which have rich in capillary blood vessels do express VEGF. In this study, we found that the expression of VEGF was closely associated with increment of the tumor grade.

The clinical course of a brain tumor may depend on relative proportion of tumor cells that are mitotically active (Rosenblum, 1989). For example, astrocytoma with a labeling index of less than 1 % manifested a slow growth rate with favorable prognosis in contrast to tumor with a higher proliferative rate (Hoshino et al., 1988). Our study also revealed the Ki-67 proliferation index was less than 1 % in group B without anaplastic progression compared to more than 3 % in group A with progression. A link between tissue bFGF and/or VEGF expression, and indices of cell proliferation has not been established previously (Casscells et al., 1990). Although a large prospective series is necessary to assess the prognostic significance of bFGF and/or VEGF expression in patients with brain tumors, it is noteworthy that there is concordance between bFGF and/or VEGF expression and Ki-67 proliferation index in our study, and a link between bFGF and/or VEGF expression and malignant pregression could be suggested.

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