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Expression of vascular endothelial growth factor in human brain tumors

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Abstract Compared to normal brain an increased expression of vascular endothelial growth factor (VEGF) has been reported in many types of brain tumors. However, the numbers of samples analyzed and information about the cellular distribution of VEGF have been limited. Here we used novel monochlonal antibodies against VEGF to analyze, using immunohistochemistry, Western blotting and enzyme-linked immunosorbent assay, its expression in 108 human brain tumors that included astrocytic tumors, meningiomas, pituitary adenomas, primary intracranial germ cell tumors and neuronal tumors. The results showed that 37 of 48 astrocytic tumors (77%) and 15 of 19 meningiomas (79%) were immunoreactive for VEGF, consistent with previous reports. However, in contrast to a previous report that analyzed only VEGF mRNA; all of our 15 pituitary adenomas showed specific immunoreactivity for VEGF. We also extended the studies to previously unanalyzed neoplasms: 13 of 15 primary intracranial germ cell tumors (82%), and 7 of 10 neuronal tumors (70%) were immunoreactive for VEGF. Direct protein analysis by Western blotting confirmed the expression of VEGF in those tumors, and showed differential expression of the isoforms of VEGF protein; a pituitary adenoma expressed both $VEGF_{165}$ and $VEGF_{189}$ proteins, a central neurocytoma expressed only $VEGF_{165}$, while an immature teratoma expressed only $VEGF_{189}$. The data herein show that VEGF is expressed in a wide spectrum of brain tumors and suggest differences among tumor entities in the mechanisms of VEGF up-regulation as well as their employment of distinct VEGF isoforms for neovascularization.

Key words Vascular endothelial growth factor · Brain tumor · Pituitary adenoma · Germ cell tumor · Neurocytoma

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Introduction

The vascular endothelial growth factor (VEGF) or vascular permeability factor, isolated from conditioned media of several types of cells [5, 14, 17], specifically promotes migration and proliferation of vascular endothelial cells, and increases vascular permeability [14, 17]. In contrast to other angiogenic factors such as the fibroblast growth factors, VEGF has a typical signal peptide, is secreted, and acts in an autocrine manner [14, 17]. Human VEGF comprises four isoforms that are generated by alternative splicing of the same mRNA, resulting in proteins of 121, 165, 189 and 206 amino acids. The two smaller isoforms are secreted from cells, while the larger two remain mostly cell associated [10].

Growth of solid tumors depends on proliferation and invasion of blood vessels from host tissue. The positive and/or negative angiogenic factors secreted by tumor cells and by other types of cells such as macrophages regulate tumor angiogenesis. Several lines of evidence have suggested that VEGF is a major positive angiogenic effector of tumors, including glioblastoma. Firstly, VEGF is expressed in glioblastoma tissues, especially in cells palisading around necrosis [23, 31]. Secondly, glioblastoma cells up-regulate VEGF expression in vitro in ischemic/hypoxic conditions, which supports the thesis that the up-

Table 1 Expression of vascular endothelial growth factor in brain tumors

Histology	*n*	No. of positive cases	No. of negative cases	% of positive cases
Astrocytic tumors	48	37	11	77
Glioblastoma	27	23	4	85
Anaplastic astrocytoma	7	5	2	71
Astrocytoma	14	9	5	64
Meningioma	19	15	4	79
Benign	11	9	2	82
Atypical/malignant	8	6	2	75
Pituitary adenoma	15	15	0	100
Non-functioning	6	6	0	100
Functioning	9	9	0	100
Neurogenic tumors	10	7	3	70
Neurocytoma	8	6	2	75
Neuroblastoma	2	1	1	50
Germ cell tumors	15	13	2	87
Germinoma	6	5	1	83
Teratomas	7	7	0	100
Other germ cell tumors	2	1	1	50

regulation of VEGF around necrosis in tumor occurs in response to hypoxia in vivo [31]. Thirdly, up-regulation of the expression of the VEGF receptors, Flt-1 and KDR, occurs in endothelial cells of glioblastoma tumors masses [23, 24]. Lastly, abrogation of VEGF expression using monoclonal antibodies or antisense mRNA suppresses angiogenicity and tumorigenicity of glioblastoma cells implanted into nude mice [3, 15, 28].

Several reports have shown that VEGF is expressed not only in glioblastomas but also in brain tumors other than glioblastoma, such as astrocytoma, ependymoma, hemangioblastoma, meningioma and metastatic brain tumors [1, 9, 20, 22, 29, 36]. Here we report the use of a new anti-VEGF monoclonal antibody, G153–694, in immunohistochemical analyses of a large number of some of these tumors as well as others that have not previously been examined. The data confirm the frequent expression of VEGF protein in astrocytic tumors and meningiomas, and extend the importance of this growth factor to pituitary adenomas, primary intracranial germ cell tumors and neuronal tumors.

Materials and methods

Brain tumor samples

One hundred and eight surgically resected brain tumor specimens were collected from the files of the Departments of Neurosurgery at the Saitama Medical School, Tokyo Metropolitan Komagome Hospital, Teikyo University Hospital, and the University of Tokyo Hospital. Forty-eight astrocytic tumors, an oligodendroglioma, 19 meningiomas, 15 pituitary adenomas, 10 neurogenic tumors and 15 primary intracranial germ cell tumors were included. All tumors were diagnosed according to the WHO classification of brain tumors [16]. Tissue samples were fixed in 10% formalin and embedded in paraffin for histological examinations. For Western blotting and enzyme-linked immunosorbent assay (ELISA), materials were snap-frozen into liquid nitrogen in operation theaters and have been kept at –80° C until use. Eleven samples were available for these assays.

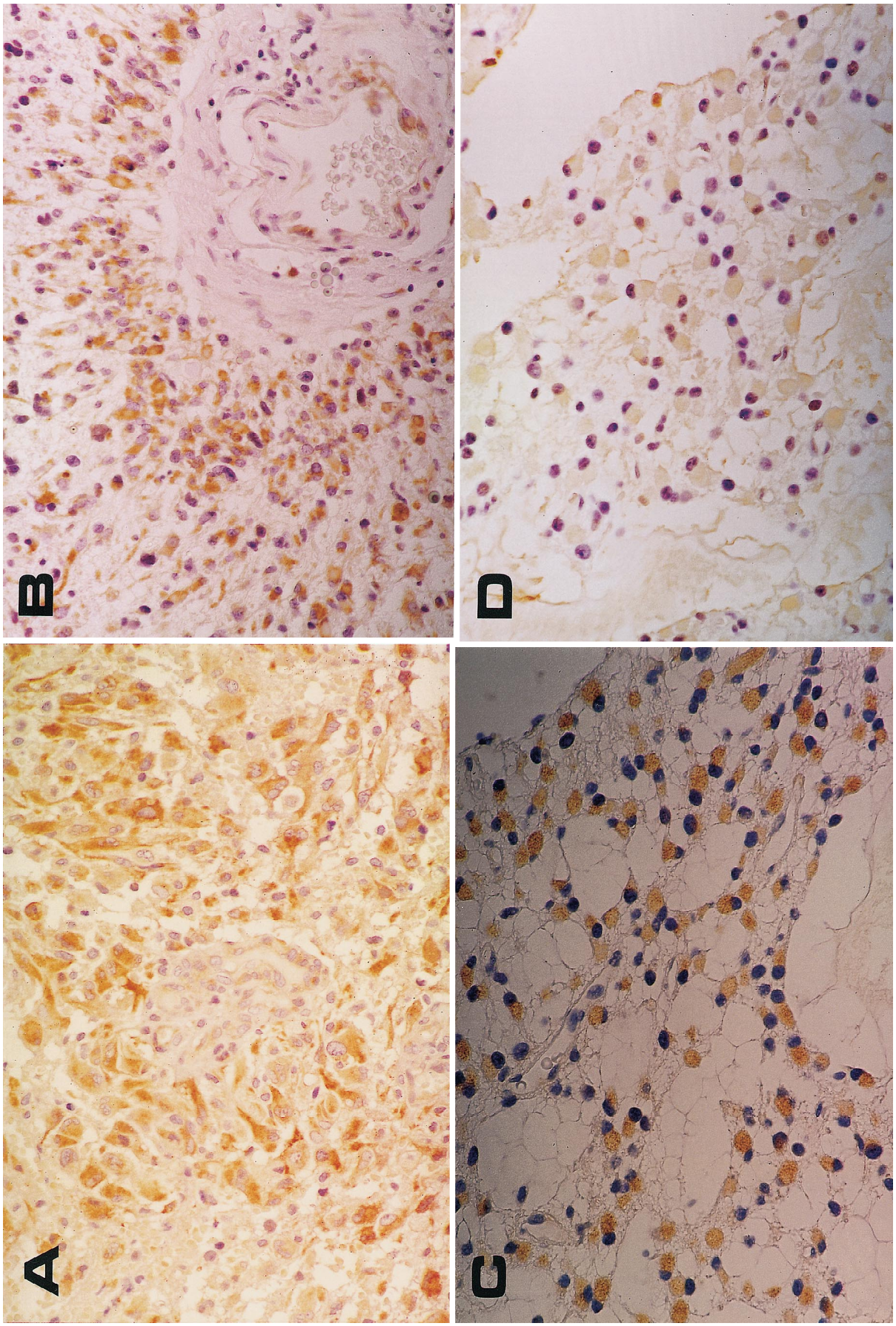
Immunohistochemistry

Sections were deparaffinized, rehydrated and incubated in hydrogen peroxide to block endogenous peroxida˜˜˜˜ctivity. An anti-VEGF monoclonal antibody raised against recombinant $VEGF_{189}$ protein (clone G153–694; PharMingen, San Diego, Calif.) [3, 22] was diluted 1 : 200 and applied to the samples for 2 h at room temperature. This antibody recognizes each of the isoforms, $VEGF_{165}$ and $VEGF_{189}$. A standard ABC method was performed according to the manufacturer's recommendations (Vectastain, Vector Lab., Burlingame, Calif.), and diaminobenzidine tetrahydrochloride was used to visualized the immunoreactivities. Slides were lightly counterstained with hematoxylin. Immunoreactive samples were defined as those in which more than 10% of the tumor cells in representative fields were stained clearly. Each specimen was also stained with mouse IgG2b (DAKO) as a negative isotype control. A glioblastoma specimen, whose expression of VEGF was ascertained using an immunoabsorption test as well as by Western blotting, was used as a positive control. Immunoabsorption tests using $VEGF_{189}$ immunogen were performed to ascertain the specificity of the antibody. Briefly, serial dilution of the $VEGF_{189}$ protein was mixed with the anti-VEGF antibody, incubated at room temperature for 60 min and applied onto specimens as for the primary antibody. Anti-S-100 protein and anti-von Willebrand factor antibodies were purchased from DAKO.

Western blotting and ELISA for VEGF

Frozen brain tumor samples were homogenized in a Dounce homogenizer containing lysis buffer, boiled, and centrifuged at

Fig. 1 A–D Representative examples of immunoreactivity for VEGF in astrocytic tumors. **A, B** Glioblastomas; **C, D** an astrocytoma. **A–C** Stained with an anti-VEGF antibody; G153–694; **D** the same sample as in **C** incubated with the antibody following preabsorption with excess amount of the immunogenic recombinant $VEGF_{189}$ polypeptide. This excess antigen dramatically reduced the immunohistochemical reactivity. All sections were counterstained with hematoxylin (*VEGF* vascular endothelial growth factor). **A, C, D** × 400, **B** × 300 ▶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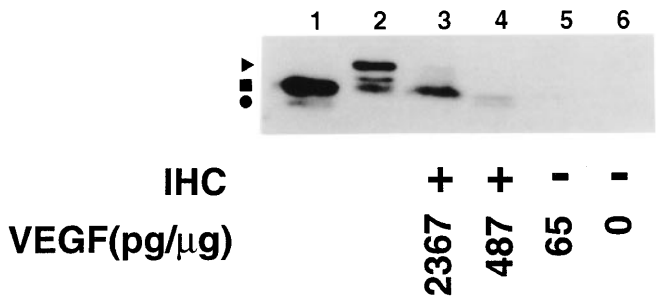


Fig. 2 Correlation of VEGF expression detected by immunohistochemistry, Western blotting and ELISA. Immunoreactivities for VEGF in IHC were indicated as + for positive and – for negative. VEGF concentration in frozen surgical samples measured by ELISA was shown in pg/μg protein of tissue samples. *Lane 1* Glioblastoma cell line, U87MG, overexpressing $VEGF_{165}$; *lane 2* U87MG overexpressing $VEGF_{189}$; *lane 3* glioblastoma whose IHC was shown in Fig. 1 A; *lane 4* astrocytoma in Fig. 1 C; *lane 5* astrocytoma negative for VEGF in IHC; *lane 6* oligodendroglioma. (▶) Glycosylated $VEGF_{189}$, (■) unglycosylated $VEGF_{189}$ or glycosylated $VEGF_{165}$, (●) unglycosylated $VEGF_{165}$ (*IHC* immunohistochemistry)

14 000 rpm at 4° C for 15 min to remove debris. The lysates were separated on an SDS/12.5% polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad). The rinsed and blocked membranes were then incubated with the mouse monoclonal anti-VEGF antibody (clone G152-341, PharMingen) at room temperature for 60 min. This anti-VEGF antibody recognizes the three isoforms of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ [4]. The blot was washed and then probed with a rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (DAKO) at room temperature for 20 min. The blot was washed again and developed with enhanced chemiluminescence reagents (Amersham). VEGF ELISA was carried out according to the manufacturer's instructions with minor modifications (Quantikine human VEGF, R & D Systems, Minneapolis, Mn). The standard curve was derived using recombinant human $VEGF_{165}$ protein (R & D Systems).

Results

Astrocytic tumors

Forty-eight astrocytic tumors including 14 cases of astrocytoma, 7 cases of anaplastic astrocytoma, and 27 cases of glioblastoma were immunohistochemically analyzed for expression of VEGF. Positive immunoreactivity was observed in 9 of 14 (64%) astrocytomas, 5 of 7 (71%) anaplastic astrocytomas, and 23 of 27 (85%) glioblastomas (Table 1). The frequencies of immunoreactive cases thus increased as the grade of astrocytic tumor progressed from astrocytoma to glioblastoma, although this was statistically not significant ($P \geq 0.1$ by a statistical test of independence), similar to a previous report [22]. Typical examples of VEGF immunoreactivity in glioblastoma are shown in Fig. 1 A, B. Constant and intensive staining, especially in tumor cells surrounding vessels with thick and degenerated endothelial walls (Fig. 1 B) was observed. Pseudopalisading cells surrounding necrosis and tumor cells around endothelial proliferation were also immunoreactive for the antibody. We observed no specific histological characteristics that could differentiate the four cases of glioblastoma negative for VEGF from those positive for VEGF: all four VEGF-negative cases of glioblastoma displayed necrosis, pseudopalisading cells, and endothelial proliferation. In cases of astrocytoma, tumor cells of gemistocytic appearances were always immunoreactive for VEGF (Fig. 1C). Immunoabsorption tests were performed using astrocytic tumors immunoreactive for VEGF. All of the immunoreactivity was dramatically reduced after absorption of the antibody in an immunogen-dose-dependent manner. A representative negative staining after absorption is shown in Fig. 1 D.

To further ascertain the specificity of this antibody, Western blotting and ELISA were performed using a VEGF-positive glioblastoma, a VEGF-positive astrocytoma, a VEGF-negative astrocytoma, and a VEGF-negative oligodendroglioma. Clear correlation between the VEGF amount indicated by ELISA, the signal intensity of the bands observed in Western blotting, and the immunohistochemical reactivity supported the specificity of this antibody (Fig. 2). The observed bands of VEGF in the astrocytic tumors migrated at 22 kDa corresponding to glycosylated $VEGF_{165}$ protein (Fig. 2, lanes 3 and 4) [4].

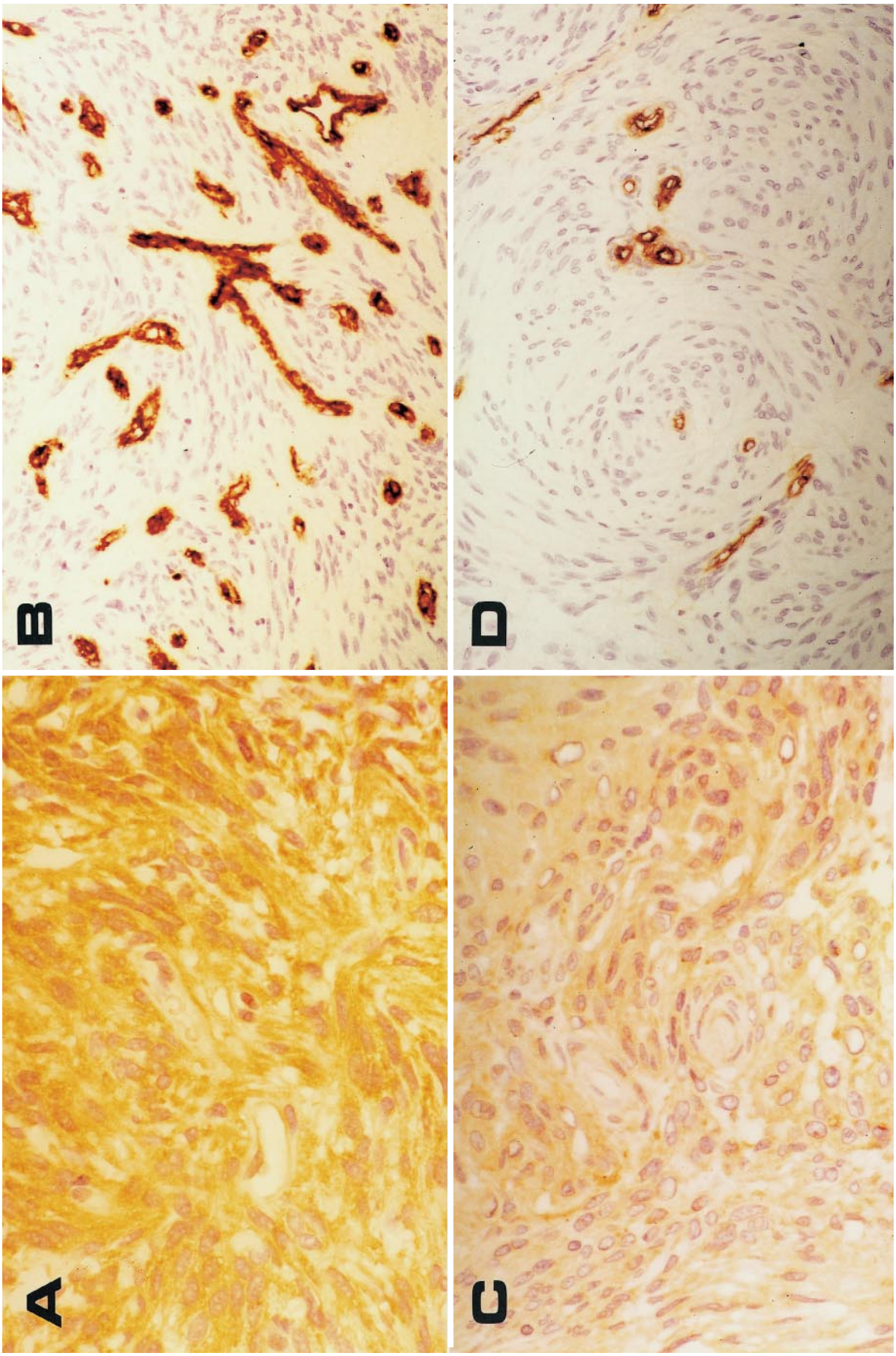
Meningiomas

Of the 19 cases of meningioma analyzed, 15 (79%) showed positive immunoreactivity for VEGF. Although we generally observed more pronounced tumor vascularities in cases of atypical or anaplastic meningiomas than in benign cases, there was no difference in frequency and distribution of VEGF immunoreactivity between meningiomas of benign histology and those of atypical or anaplastic histology: 9 of 11 (82%) benign meningiomas and 6 of 8 (75%) atypical or anaplastic meningiomas were immunoreactive for VEGF. This lack of association between the expression of VEGF and the histopathological subtypes of meningiomas is similar to a previous report [22]. The most intensive immunoreactivity for VEGF was observed in a highly vascular case shown in Fig. 3 A, whose anti-von Willebrand factor staining is shown in Fig. 3 B. Benign meningiomas with positive VEGF immunoreactivity were not necessarily hyper-vascular (VEGF and von Willebrand factor staining of a representative benign meningioma case are shown in Fig. 3 C and D, respectively). Atypical or anaplastic meningiomas with negative VEGF expression were not hypo-vascular (data not shown).

Pituitary adenomas

Each of the 15 cases of pituitary adenomas analyzed were immunoreactive for VEGF. Nine adenomas were func-

Fig. 3 A–D VEGF expression and vascularity in meningiomas. **A, B** Meningothelial meningioma with high vascularity; **C, D** meningothelial meningioma with low vascularity. **A, C** VEGF immunoreactivity; **B, D** von Willebrand factor immunoreactivity showing tumor vessels. **A, C** × 600, **B, D** × 300 ▶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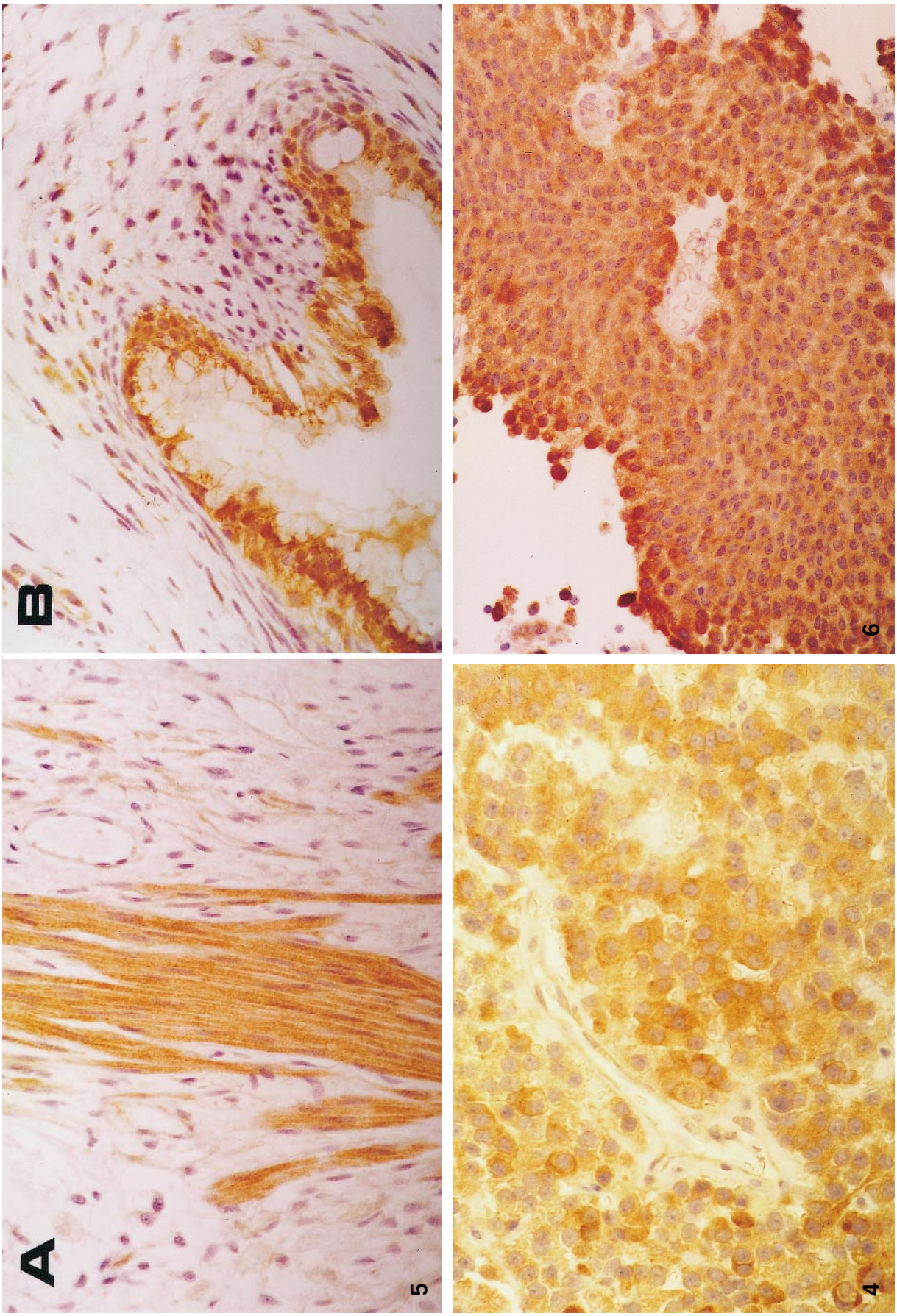
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tioning; 4 cases were secreting growth hormone, 2 were secreting prolactin, 2 were secreting adrenocorticotropic hormone, and 1 was secreting thyroid-stimulating hormone. The other 6 cases were non-functioning adenomas. A representative case is shown in Fig. 4. Although the distribution of VEGF-immunoreactive cells was diffuse, some tumor cells presented prominent immunoreactivity, as shown in Fig. 4. There was no difference in distribution of immunoreactive tumor cells or intensity of staining between the functioning and non-functioning adenomas. VEGF was first purified from conditioned media of bovine pituitary folliculostellate cells [17]. Pituitary folliculostellate cells are known to show conspicuous and strong reaction with anti-S-100 protein antibody [21]. To demonstrate that these VEGF-reactive cells are not folliculostellate cells entangled in the tumors, we determined the immunoreactivity for S-100 protein; all sections were negative except for the compressed normal pituitary tissue surrounding tumors (data not shown).

Primary intracranial germ cell tumors

Primary intracranial germ cell tumors are believed to originate from primordial germ cells [30]. Their apparent geographical incidence and increased frequency in Japan provided us with an opportunity to analyze these tumors that are relatively rare in the Western countries [27]. A series of 15 primary intracranial germ cell tumors were studied for VEGF immunoreactivity: 6 cases of germinoma, 6 immature and 1 mature teratomas, 1 choriocarcinoma and 1 yolk sac tumor were included. Five of the 6 germinomas (83%), all the 7 teratomas (100%), and the yolk sac tumor were immunoreactive for VEGF. In total, 13 of 15 (87%) primary intracranial germ cell tumors expressed VEGF. In the VEGF-positive cases of germinoma and yolk sac tumor, immunoreactive tumor cells distributed diffusely and more than 50% of the tumor cells were positive for VEGF. In the cases of teratoma, strong immunoreactivity was restricted to cells showing certain types of tissue characteristics, such as muscular, gastrointestinal, bronchial, and transitional epithelial differentiation as shown in Fig. 5 A, B. Immunohistochemistry using anti-von Willebrand factor antibody revealed that tumor vascularity was minimal even in those areas containing VEGF-positive cells in the teratomas (data not shown).

Neuronal tumors

We analyzed 10 cases of intracranial neuronal tumors; 8 cases of central neurocytoma, 1 oldfactory neuroblastoma and 1 intracranial metastatic neuroblastoma. Positive immunoreactivity was observed in 6 of 8 of the neurocytomas (75%) and in the metastatic neuroblastoma. The distribution of immunoreactive cells was diffuse and homogeneous as shown in Fig. 6.

◀ **Fig. 4** Expression of VEGF in a pituitary adenoma. × 400

Fig. 5 A, B Expression of VEGF in a primary intracranial immature teratoma. Immunoreactive cells in **A** and **B** present smooth muscular and possible gastric epithelial differentiation with goblet cells, respectively. **A, B** × 400

Fig. 6 Expression of VEGF in a central neurocytoma, × 400

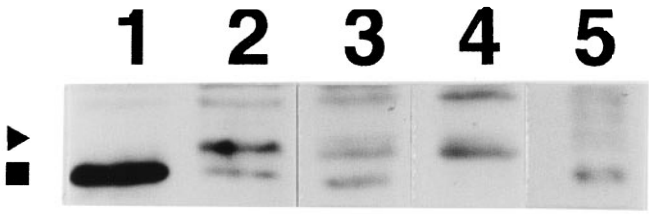

Fig. 7 VEGF Western blotting of a pituitary adenoma, a primary intracranial germ cell tumor (an immature teratoma), and a central neurocytoma. *Lane 1* glioblastoma cell line, U87MG, overexpressing $VEGF_{165}$; *lane 2* U87MG overexpressing $VEGF_{189}$; *lane 3* pituitary adenoma whose immunoreactivity was shown in Fig. 4; *lane 4* immature teratoma shown in Fig. 5; *lane 5* central neurocytoma shown in Fig. 6. (▶) Glycosylated $VEGF_{189}$, (■) unglycosylated $VEGF_{189}$ or glycosylated $VEGF_{165}$. The bands with larger molecular size than that of the VEGF-specific bands marked are nonspecific cross-reactions with the antibody that serve as internal loading control

Western blotting

Representative frozen samples of pituitary adenoma, immature teratoma and neurocytoma (from the same cases whose immunohistochemical findings are shown in Figs. 4, 5 A and B, and 6, respectively) were prepared for Western blotting. A pituitary adenoma sample had both 22- and 28-kDa VEGF species (Fig. 7, lane 3) corresponding to glycosylated $VEGF_{165}$ and glycosylated $VEGF_{189}$, respectively [4]. An immature teratoma sample displayed a significant amount of 28-kDa $VEGF_{189}$ protein, but not the 22-kDa $VEGF_{165}$ species (Fig. 7, lane 4). A neurocytoma expressed predominantly 22-kDa $VEGF_{165}$ (Fig. 7, lane 5).

Discussion

Since the first report of VEGF-up-regulated expression in glioblastoma [23], this has also been demonstrated in a number of other brain tumors including astrocytomas, hemangioblastomas, meningiomas and metastatic brain tumors [1, 9, 20, 22, 29, 36]. However, histopathological resolution and the numbers of samples analyzed have been limited, as most of those studies employed in situ hybridization and Northern blotting techniques. Recently, a novel monoclonal antibody for VEGF, G153–694, was developed, which stains paraffin-embedded sections well [3], and 173 brain tumors have been analyzed by immunohistochemistry and Western blotting using it [22]. Ependymoma was found to express VEGF frequently, while 100% of oligodendrogliomas and 76% of medulloblastomas did not. Here, we confirmed and extended

some of the findings on astrocytic tumors and also analyzed pituitary adenomas, primary intracranial germ cell tumors and neuronal tumors.

Similar to the previous reports which analyzed VEGF mRNA expression [1, 9, 23, 29, 35], glioblastoma expressed VEGF protein frequently. In contrast to the in situ hybridization studies on the distribution of VEGF mRNA [9, 23, 24, 31], we did not detect a predominant localization of the VEGF protein in pseudopalisading tumor cells surrounding necrotic area. The distribution of immunoreactive cells was diffuse including pseudopalisading cells. This discrepancy was probably due to the different sensitivities of the assays or to the different stability of VEGF mRNA and protein. In the previous immunohistochemical studies, VEGF immunoreactivity was strongest in the vasculature [23, 24]. Our study observed intensive staining in tumor cells surrounding vessels but not in the endothelial cells (Fig. 1 B). This difference could be because of the difference of the antibodies used.

The level and frequency of VEGF expression in astrocytomas have been reported to be low [24, 29, 33], possibly reflecting the relatively less angiogenicity of these tumors compared to glioblastoma. A significant correlation of VEGF RNA expression and vascularity in gliomas including astrocytomas has been reported [29]. However, here and previously [22], it was observed that 60–70% of cases of astrocytomas were immunoreactive for VEGF. This may be due to a greater sensitivity of the methodology or because VEGF levels can be regulated not by increasing mRNA levels, but by stabilizing them. Stabilization of VEGF mRNA has been reported under hypoxic conditions [12, 18, 32]. As hypoxia in astrocytoma has not been confirmed, other factors may be contributing to the VEGF mRNA stabilization. Astrocytomas immunoreactive for VEGF were not necessarily hypervascular estimated by anti-von Willebrand factor antibody staining. As the VEGF receptors (Flt-1 and KDR) were reported to be co-expressed in endothelial cells in glioblastoma but not in astrocytoma [24], it may be that the VEGF expressed in relatively hypovascular astrocytomas play functions other than in angiogenesis, such as the induction of vascular permeability. It is interesting that cystic fluids in glioblastoma also contained high amounts of VEGF protein [1, 33, 35]. Microcystic degeneration is one of the characteristic features in protoplasmic astrocytomas [27], and our cases of astrocytoma with abundant cystic components had strongly up-regulated VEGF levels (Fig. 1 D). This may suggest that VEGF contributes to increased vascular permeability and cyst formation in astrocytomas, although further studies are necessary to prove this point.

Our present results agree with previous reports that VEGF is frequently expressed in meningiomas [1, 9, 22]. In our series of meningioma, the most intense immunoreactivity was observed in a highly vascular case (Fig. 3 A, B). This observation corresponds well with previous reports suggesting a correlation between VEGF expression and vascularity in meningiomas [9, 29]. High levels of VEGF receptor (Flk-1) expression in meninges have also been observed in mouse development [19], suggesting that the meningeal arachnoidal cells from which meningiomas originate up-regulate VEGF and, in turn, trigger tumor vascularization derived from meningeal vessels. On the other hand, some cases of meningioma immunoreactive for VEGF were relatively hypovascular as shown in Fig. 3 C, D. Recent reports showed that meningiomas with a large amount of peritumoral edema had elevated expression levels of VEGF, and suggested that VEGF expression is an important determinant for edema formation in meningiomas [13, 25].

All of the cases of pituitary adenomas we analyzed were diffusely immunoreactive for VEGF, and Western blotting data supported this interpretation (Fig. 7, lane 3). In constrast, low frequencies of VEGF expression in pituitary adenomas (2/10 = 20%) have been detected using Northern blotting technique [1], and this was suggested to be due to the low levels of tumor vascularity and peritumoral edema in this type of tumor. However, pituitary adenomas, especially those of sinusoidal or papillary type, have well-developed capillary networks characteristic of endocrine tumors [26]. Diffuse type pituitary adenomas also have intersecting connective tissue stroma that contains capillaries stained with antibodies to the von Willebrand factor (data not shown). Therefore, the VEGF protein expressed in pituitary adenomas may actually play a significant role in their angiogenesis. The negative immunoreactivities for S-100 protein in these tumors minimized the possibility that the immunoreactive cells found in pituitary adenomas were folliculostellate cells [11]. Recently, tumor-associated intracerebral hemorrhage caused by overexpression of VEGF in glioblastoma cells was reported [4], and intratumoral hemorrhage is the most frequent in pituitary adenomas among all brain tumors [34], perhaps suggesting an etiological association.

High frequencies of VEGF expression were also observed in primary intracranial germ cell tumors. Immature teratomas showed immunoreactivity for VEGF predominantly in cells displaying differentiation into smooth muscle cells, bronchial, gastrointestinal and transitional epitheliums. VEGF is known to be expressed by a number of normal tissues including cardiac myocytes, vascular and gastrointestinal smooth muscle, lung alveolar epithelium, stomach and colon mucous epithelium, and tumors derived from those tissues (reviewed in [6]). A high proportion of VEGF-positive tumors was identified among germinomas, consistent with the expression of VEGF at high levels in many fetal tissues, including seminiferous tubules of testis [2]. In the single case of immature teratoma we could analyze by Western blotting, the major species of VEGF protein was $VEGF_{189}$ (Fig. 7, lane 5), while the major species of VEGF protein expressed in other brain tumors was $VEGF_{165}$. The observed ratios of $VEGF_{165}$ expression to $VEGF_{189}$ expression were different in various organs at differentiation stages [2, 7, 8]. During avian embryogenesis, $VEGF_{190}$ that is homologous to the human $VEGF_{189}$ was most abundant in heart and liver, while $VEGF_{166}$, homologue of the human $VEGF_{165}$, was the most abundant species in kidney and brain [7, 8]. Up-regulation of $VEGF_{189}$ in teratomas may reflect its differentiation

situation, although more detailed studies are warranted. Nonetheless, it is relevant to note that $VEGF_{121}$ and $VEGF_{165}$, but not $VEGF_{189}$, could cause experimental hemorrhage [4], suggesting that these isotype expression differences may have biological relevance. Among the 11 brain tumors analyzed by Western blotting, we observed a single case of glioblastoma that expressed a faint band corresponding to unglycosylated $VEGF_{165}$ or glycosylated $VEGF_{121}$ (data not shown). Studies on mRNA expression have suggested that $VEGF_{121}$ was the second abundant isoform of VEGF in brain tumors [1]. As the numbers of cases studied by Western blotting was small, the significance of the differential expression of these isoforms in brain tumors remains to be elucidated.

Frequent expression of VEGF in neuronal tumors was another novel observation of our studies. During embryonic brain development, VEGF transcripts were abundant in the ventricular neuroectoderm, where neuron and glia originate and differentiate, but were reduced in the adult brain [2]. Up-regulation of VEGF in primitive neuroectoderm may suggest that neuronal cells have the potential to up-regulate VEGF when they are proliferating. If so, the VEGF up-regulation observed in neuronal tumors is understandable, as the tumor cells are aggressively proliferating and require vascularization.

To date, oligodendroglioma, pilocytic astrocytoma, and medulloblastoma are the only brain tumors for which less than 50% of the cases have been shown to be immunoreactive for VEGF [22]. In our study, we did not include these tumors except for a single case of oligodendroglioma that was negative for VEGF. In those tumors, angiogenic factors other than VEGF are likely contributing to their vascularization. Our findings showed that VEGF protein is expressed not only in brain tumors rich in vascularity such as glioblastoma, but also in less vascular tumors such as astrocytomas, meningiomas, and intracranial teratomas. In astrocytomas and meningiomas, VEGF may contribute to cyst formation and peritumoral edema. Up-regulation of VEGF in neuronal tumors and primary intracranial germ cell tumors may reflect its role in cognate tissue development. More detailed analyses to elucidate the molecular mechanisms of VEGF up-regulation are necessary for further understanding of brain tumor genesis and progression, and for possible therapeutic application of VEGF.

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References

1. Berkman RA, Merrill MJ, Reinhold WC, Monacci WT, Saxena A, Clark WC, Robertson JT, Ali IU, Oldfield EH (1993) Expression of the vascular permeability factor/vascular endothelial growth factor gene in central nervous system neoplasms. J Clin Invest 153–159
2. Breier G, Albrecht U, Sterrer S, Risau W (1992) Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development 114: 521–532
3. Cheng S-Y, Su Huang H-J, Nagane M, Ji X-D, Wang D, Shih CC-Y, Arap W, Huang C-M, Cavenee WK (1996) Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. Proc Natl Acad Sci USA 93: 8502–8507
4. Cheng S-Y, Nagane M, Su Huang H-J, Cavenee WK (1997) Intracerebral tumor-associated hemorrhage caused by overexpression of the vascular endothelial growth factor isoforms VEGF121 and VEGF165 but not VEGF189. Proc Natl Acad Sci USA 94: 12 081–12 087
5. Conn G, Soderman DD, Schaeffer M-T, Wile M, Hatcher VB, Thomas KA (1990) Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. Proc Natl Acad Sci USA 87: 1323–1327
6. Dvorak HF, Brown LF, Detmar M, Dvorak AM (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 146: 1029–1039
7. Flamme I, Breier G, Risau W (1995) Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. Dev Biol 169: 699–712
8. Flamme I, Reutern M von, Drexler HCA, Syed-Ali S, Risau W (1995) Overexpression of vascular endothelial growth factor in the avian embryo induces hypervascularization and increased vascular permeability without alterations of embryonic pattern formation. Dev Biol 171: 399–414
9. Hatva E, Kaipainen A, Mentula P, Jääskeläinen J, Paetau A, Haltia M, Alitalo K (1995) Expression of endothelial cell-specific receptor tyrosine kinases and growth factor in human brain tumors. Am J Pathol 146: 368–378
10. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW (1991) The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 5: 1806–1814
11. Höfler H, Walter GF, Denk H (1984) Immunohistochemistry of folliculostellate cells in normal human adenohypophyses and in pituitary adenomas. Acta Neuropathol (Berl) 65: 35–40
12. Ikeda E, Achen MG, Breier G, Risau W (1995) Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. J Biol Chem 270: 19 761–19 766
13. Kalkanis SN, Carroll R, Zhang J, Zamani AA, Black PM (1996) Vascular endothelial growth factor (VEGF) expression correlates with increased peritumoral vasogenic cerebral edema in meningiomas. J Neurosurg 85: 1095–1101
14. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246: 1309–1312
15. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 362: 841–844
16. Kleihues P, Burger PC, Scheithauer BW (1993) Histological typing of tumours of the central nervous system. Springer, New York Berlin Heidelberg
17. Leung DW, Cachianes G, Kuang W-J, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246: 1306–1309

18. Levy AP, Levy NS, Wegner S, Goldberg MA (1995) Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J Biol Chem 270 : 13 333–13 340
19. Millauer B, Wizigmann-Voos S, Schnürch H, Martinez R, Møller NP, Risau W, Ullrich A (1993) High affinity VEGF binding and developmental expression suggest flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72 : 835–846
20. Morii K, Tanaka R, Washiyama K, Kumanishi T, Kuwano R (1993) Expression of vascular endothelial growth factor in capillary hemangioblastoma. Biochem Biophys Res Commun 194 : 749–755
21. Nakajima T, Yamaguchi H, Takahashi K (1980) S-100 protein in folliculostellate cells of the rat pituitary anterior lobe. Brain Res 191 : 523–531
22. Pietsch T, Valter MM, Wolf HK, Deimling A von, Huang H-JS, Cavenee WK, Wiestler OD (1997) Expression and distribution of vascular endothelial growth factor protein in human brain tumors. Acta Neuropathol 93 : 109–117
23. Plate KH, Breier G, Weich HA, Risau W (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359 : 845–848
24. Plate KH, Breier G, Weich HA, Mennel HD, Risau W (1994) Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. Int J Cancer 59 : 520–529
25. Provias J, Claffey K, DelAguila L, Lau N, Feldkamp M, Guha A (1997) Meningiomas: role of vascular endothelial growth factor/vascular permeability factor in angiogenesis and peritumoral edema. Neurosurgery 40 : 1016–1026
26. Rubinstein LJ (1981) Tumors of the central nervous system. Armed Forces Institute of Pathology, Washington DC
27. Russell DS, Rubinstein LJ (1989) Pathology of tumours of the nervous system, 5th edn. Edward Arnold, London
28. Saleh M, Stacker SA, Wilks AF (1996) Inhibition of growth of C6 glioma cells in vivo by expression of antisense vascular endothelial growth factor sequence. Cancer Res 56 : 393–401
29. Samoto K, Ikezaki K, Ono M, Shono T, Kohno K, Kuwano M, Fukui M (1995) Expression of vascular endothelial growth factor and its possible relation with neovascularization in human brain tumors. Cancer Res 55 : 1189–1193
30. Sano K, Matsutani M, Seto T (1989) So-called intracranial germ cell tumors: personal experiences and a theory of their pathogenesis. Neurol Res 11 : 118–126
31. Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359 : 843–845
32. Stein I, Neeman M, Shweiki D, Itin A, Keshet E (1995) Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. Mol Cell Biol 15 : 5363–5368
33. Takano S, Yoshii Y, Kondo S, Suzuki H, Maruno T, Shirai S, Nose T (1996) Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients. Cancer Res 56 : 2185–2190
34. Wakai S, Yamakawa K, Manaka S, Takakura K (1982) Spontaneous intracranial hemorrhage caused by brain tumors: its incidence and clinical significance. Neurosurgery 10 : 437–444
35. Weindel K, Moringlane JP, Marme D, Weich HA (1994) Detection and quantification of vascular endothelial growth factor/vascular permeability factor in brain tumor tissue and cyst fluid: the key to angiogenesis? Neurosurgery 35 : 439–448; discussion 448–449
36. Wizigmann-Voos S, Breier G, Risau W, Plate KH (1995) Upregulation of vascular endothelial growth factor and its receptors in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. Cancer Res 55 : 1358–1364