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Fumihide Yoshimura · Toshiyuki Kaidoh
Tetsuo Inokuchi · Minoru Shigemori

Changes in VEGF expression and in the vasculature during the growth of early-stage ethylnitrosourea-induced malignant astrocytomas in rats

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Abstract Vascular endothelial growth factor (VEGF), a potent angiogenic and vascular permeability factor, may be important as a mediator of brain tumour progression. However, it is still not clear whether VEGF plays a causative role in the early stage of glioma development. We investigated the relationship between VEGF protein expression (as assayed by immunohistochemistry) and different morphological parameters reflecting tumour progression (tumour diameter, vascular density and vascular diameter) in tumours at various stages. As a tumour model, ethylnitrosourea (ENU)-induced rat malignant astrocytoma was used. Tumours were classified by size and level of vascularity estimated by the von Willebrand factor (vWF) staining. Tumours less than 10 mm in diameter were designated early stage neoplastic lesions. All 34 early astroglial tumours were found to be VEGF positive. Increase in the VEGF immunopositive rate of tumour cells correlated significantly with increase in vascular density and vascular diameter. We suggest that VEGF induces angiogenesis and growth of microvessels, promoting growth of the early stage malignant astrocytoma.

Key words Vascular endothelial growth factor (VEGF) · Ethylnitrosourea (ENU) · Angiogenesis · Malignant astrocytoma

F. Yoshimura (✉) · M. Shigemori
Department of Neurosurgery,
Kurume University School of Medicine, 67 Asahi-machi,
Kurume, 830, Japan
Tel.: +81-942-31-7570, Fax: +81-942-38-8179

T. Kaidoh[1]
Department of Anatomy, Kurume University School of Medicine,
Kurume, Japan

T. Inokuchi
Department of Anatomy, Kurume University School of Medicine,
Kurume, Japan

Present address:
[1] Department of Anatomy, Faculty of Medicine, Tottori University,
Tottori, Japan

Introduction

Solid tumours induce vascular proliferation by means of angiogenic factors; the vascular proliferation supports growth of the tumours [8, 15]. Vascular endothelial growth factor (VEGF) [7], a potent angiogenic agent, plays an important part in angiogenesis in various tumours [4, 28], and brain tumours in particular. There have been several reports on the presence of VEGF mRNA in tumour cells within human brain [16, 20, 25]. Significant elevation of *VEGF* gene expression has been observed in highly vascular human CNS neoplasms, and a significant role is suggested for VEGF in the development of neovascularity in CNS tumours [2]. Northern blot analysis of several growth factors in human gliomas and meningiomas has shown that the expression of the *VEGF* gene is closely correlated with vascularity in both tumours, but that of other angiogenic factor genes, such as the *bFGF*, *TGF-β*, and *TGF-α* genes, is not [23].

Localization of the VEGF protein has been reported in surgically obtained human brain tumour tissues [20, 21, 27]. In the reports of this, a VEGF-positive reaction was observed in tumour cells producing VEGF or in vasculature where VEGF exerts its biological action.

In the early stage of tumour growth the onset of vascularization is a critical step in the start of rapid growth. Intraoc ular tumour implant models show that tumours stop growing at 1–2 mm^3, but resume rapid growth when vascularization is permitted [9]. Since the VEGF expression is correlated with vascularity in surgically removed adult human brain tumours [2, 19, 23, 27], it is likely that VEGF also plays a key role in vascularization of early-stage brain tumours. However, it is not clear whether or not early tumours actually express VEGF and, if so, whether VEGF-induced angiogenesis in turn promotes growth. To answer these questions, we assessed the VEGF-positive rate of the tumour cells [VEGF(+)rate], tumour diameter, vascular density, and vascular diameter in ethylnitrosourea (ENU)-induced rat brain tumours. This experimental brain tumour is a representative model for human tumours of the nervous

system [3] and provides early-stage tumours (less than 10 mm in diameter).

We also immunostained von Willebrand factor (vWF; antibleeding factor or coagulation factor VIII A) a useful marker for brain tumour microvessels [17, 24] to separate the brain tumours with vWF-positive tumour microvessels from those without such microvessels. Based on vWF immunostaining and tumour size, tumours were classified into three groups: small tumours less than 1 mm in diameter, medium-sized tumours 1–2.5 mm in diameter, and large tumours more than 2.5 mm in diameter. Statistical comparisons were then made of the VEGF(+)rate, vascular density, and vascular diameter of these groups.

Materials and methods

This study was carried out after permission had been granted by the Committee of Animal Experimentation, Kurume University.

Intracranial neoplasms were induced transplacentally in six pregnant Sprague-Dawley (SD) rats on day 14 or 15 of gestation, by a single intraperitoneal administration of ENU (50 mg/kg body weight). Of the 41 offspring, 29 offspring with motor palsy were used for this study 150–180 days after birth. Motor palsy is one of the major symptoms of this brain tumour. Under sodium pentobarbital anaesthesia, the animals were successively perfused with heparinized (4 unit/ml) saline and 4% formaldehyde solution in 0.1 M phosphate buffer (pH 7.4). The brain tissues were removed, immersed in the same fixative overnight at 4°C, and sliced coronally at a thickness of 2 mm to investigate the presence of tumours. Tissue slices were immersed in 0.1 M phosphate buffer containing 20% sucrose solution, embedded in OCT medium, rapidly frozen in dry ice-ethanol, and stored at –80°C.

The pathological classification of the ENU-induced rat brain tumours was performed on H&E-stained cryosections. The WHO classification of human brain tumours was not always suitable for classification of the rat brain tumours because they were usually composed of a mixed cell population. This consisted mainly of astroglioma, oligodendroglioma and anaplastic glioma cells [3]. In the study, we used tumours that consisted mainly of astroglioma cells and/or anaplastic glioma cells and designated these astroglial tumours malignant astrocytomas. Tumours that consisted mainly of oligodendroglioma cells, schwannoma cells, ependymoma cells, or neurinoma cells were excluded.

The cryostat serial sections (8 µm) were mounted on silan-coated slide glasses and air-dried for 1 h. In each tissue block, one of the serial sections was stained with haematoxylin and eosin (H&E) and the longest diameter of the tumour was measured using a graticule. In each tumour section, 10 fields (each field 0.1 mm^2) were randomly selected, the number of blood vessels in each field was scored, and the number counted in all fields was calculated as the vascular density per tumour area. In all small tumours and some medium-sized tumours, however, the 10 fields were randomly selected from several sections because the area in one section was smaller than the sum area of the 10 fields. The number of blood vessels was scored and the vascular density was calculated in the same manner in the normal cerebral tissues.

In each tumour section, 10 blood vessels were randomly selected and the minor axis of each blood vessel that was regarded as its vascular diameter was measured using a graticule. The mean of the vascular diameters was calculated in each tumour. The vascular diameter was also measured in the normal cerebral tissues.

Sections adjacent to H&E-stained sections were used for immunohistochemical staining of VEGF. These sections were initially blocked for 1 h at room temperature in 0.1 M phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 2.5% normal goat serum (NGS), and 0.01% Triton X-100, and then incubated in primary antibody (affinity-purified rabbit anti-VEGF; diluted 1:800 in the blocking solution; Santa Cruz Biotechnology) for 48 h at 4°C. The sections were washed with 0.1 M PBS containing 0.1% BSA (PBSA) and incubated in biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories) diluted 1:400 in PBSA containing 2.5% NGS for 1 h at room temperature. After a further wash in PBSA, the sections were treated with avidin–biotin complex (Vectastain ABC kit, Vector Laboratories), washed in PBS, and then incubated in cobalt-nickel-diaminobenzidine-H_2O_2 solution for 5 min [1]. The sections were dehydrated in increasing concentrations of ethyl alcohol, cleared in xylene, and mounted. The control experiments were carried out by omission of the primary antibody and by incubation of the sections with the primary antibody and its control peptide (Santa Cruz Biotechnology).

Sections adjacent to H&E-stained sections were used for immunohistochemical staining of vWF. These sections were initially blocked for 1 h at room temperature in 0.1 M PBS containing 1% BSA and 2.5% NGS, and then incubated in primary antibody (affinity-purified rabbit anti vWF; diluted 1:1000 in blocking solution; DAKO) for 1 h at room temperature. The sections were washed with 0.1 M PBS and incubated in biotinylated goat anti-rabbit immunoglobulin diluted 1:400 in PBSA containing 2.5% NGS for 1 h at room temperature. After a further wash in PBSA, the sections were treated with avidin–biotin complex, washed in PBS, and then incubated in 0.02% diaminobenzidine and 0.01% H_2O_2 in Tris buffer saline solution for 5 min. The sections were dehydrated and mounted. The control experiments were carried out by omission of the primary antibody.

VEGF-immunostained tissue specimens were analysed and scored by two different observers. In each tissue specimen, 10 fields (each field: 0.016 mm^2) were examined under light microscopy. The intensity of VEGF immunoreactivity of each tumour cell was evaluated as negative, weakly positive or positive. Tumour cells with weakly positive and positive immunoreactivity were regarded as VEGF-positive tumour cells. Total tumour cells and VEGF-positive tumour cells were then counted, and the percentage of VEGF-positive tumour cells among the total tumour cells was regarded as the VEGF(+)rate. The result of the VEGF staining was compared statistically with tumour size, tumour vascularity, and vascular diameter in the tumour tissue.

Slides immunostained for vWF were analysed and scored by two different observers. According to the vWF immunoreactivity, tumour microvessels were graded as negative, positive, and strongly positive.

Results

The pathological classification of the ENU-induced rat brain tumours is shown in Table 1. Thirty-four malignant astrocytomas that were less than 10 mm (0.5–9 mm) in diameter consisted mainly of undifferentiated astrocytes characterized by a high nuclear cytoplasmic ratio and a few short cytoplasmic processes (Fig. 1); these were ex-

Table 1 Incidence and classification of ENU-induced tumours of the nervous system. Malignant astrocytomas consisted mainly of astroglioma cells and/or anaplastic glioma cells. We used 34 malignant astrocytomas that were less than 10 mm in diameter

	No.
Malignant astrocytoma	44
<10 mm	34
≥10 mm	10
Oligodendroglioma	13
Medulloblastoma	3
Schwannoma	5
Ependymoma	2
Neurinoma	2
Total	69

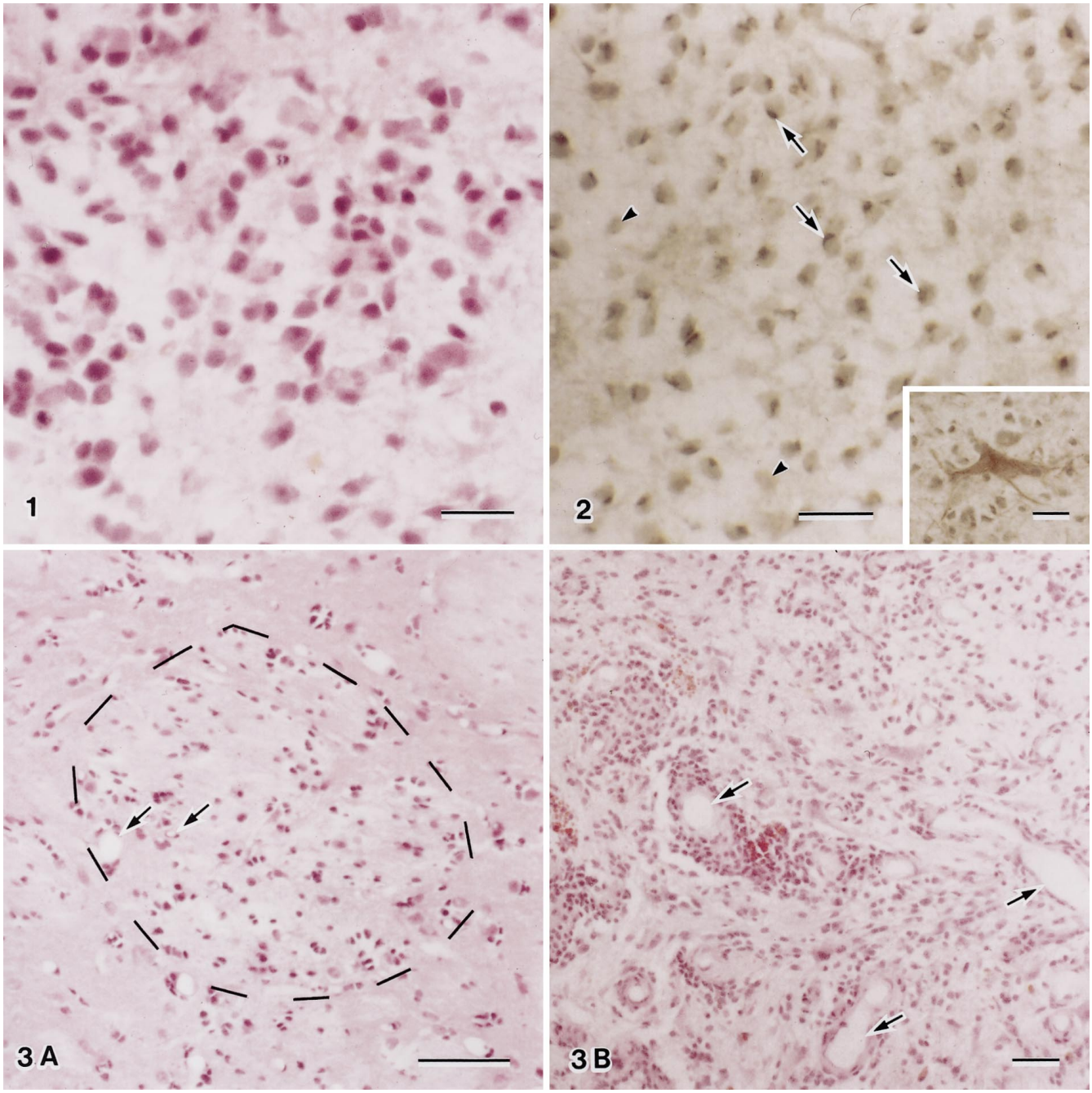


Fig. 1 Ethylnitrosourea (ENU)-induced malignant astrocytoma. Tumour cells show a high nuclear cytoplasmic ratio and a few short processes. H&E, *bar* 20 µm

Fig. 2 Vascular endothelial growth factor (VEGF) immunostaining of the tumour tissue. Tumour cells show positive reaction as a dark brown precipitate (*arrows*) in their cytoplasm near the nucleus. VEGF-negative tumour cells (*arrowheads*). *Inset* A reactive astrocyte with a large polygonal cell body shows positive reaction in their cytoplasm. *Bar* 20 µm

Fig. 3 ENU-induced **A** small and **B** large malignant astrocytoma **A** *Arrows* show intratumour blood vessels. A *broken line* is a border between the tumour tissue and the surrounding brain tissue. **B** Arrows show intratumour blood vessels with dilatation.H&E, *bar* 50 µm

amined for VEGF immunoreactivity, vascular density, vascular diameter, and vWF immunoreactivity.

All 34 malignant astrocytomas, including those less than 1 mm in diameter, showed a VEGF-positive immunoreaction (Fig. 2). VEGF-positive cells were mainly tumour cells. VEGF-positive reaction in tumour cells was located in their cytoplasm adjacent to the cell nucleus (Fig. 2). The VEGF-positive tumour cells appeared to be distributed uniformly throughout the tumour, including the periphery of the necrotic area. A small number of vascular endothelial cells and reactive glial cells also showed VEGF-positive immunoreactivity.

Since VEGF immunoreactivity was different in each tumour, we assessed the VEGF(+)rate, which varied from

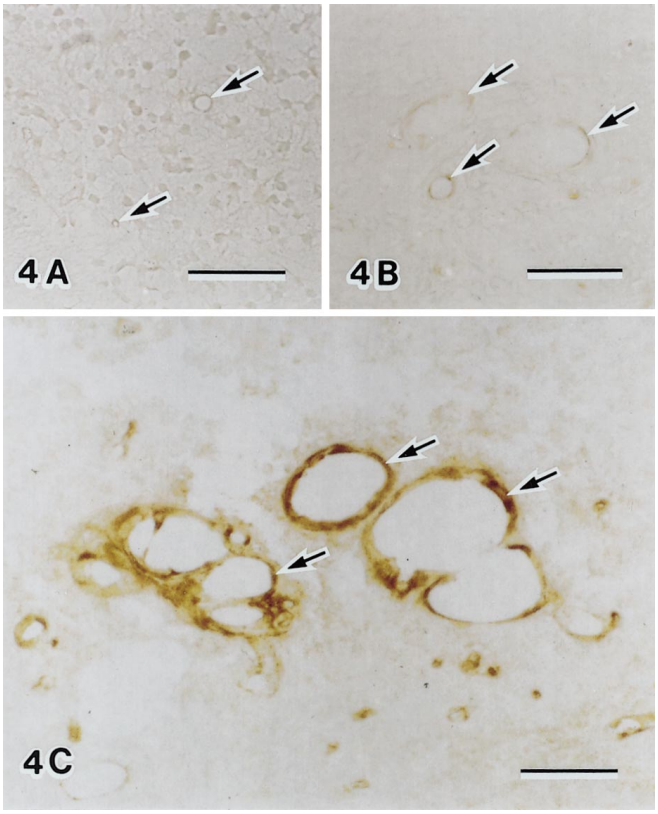


Fig. 4A–C von Willebrand factor (vWF) immunostaining of the tumour tissue. **A** Blood vessels without positive immunoreaction in the small tumour (*arrows*). **B** Blood vessels with positive immunoreaction in the medium-sized tumour (*arrows*). **C** Dilated blood vessels with strong positive immunoreaction in the large tumour (*arrows*). *Bar* 50 µm

39.0% to 87.8%. Of 17 tumours that were more than 2.5 mm in diameter, 11 had a high (>70%) VEGF(+)rate. In contrast, all 17 tumours that were less than 2.5 mm in diameter had VEGF(+)rates less than 70%.

All 34 malignant astrocytomas contained microvessels. In individual tumours, the vascular density varied from 8.7 to 19.0, and the vascular diameter varied from 5.3 to 51.8 µm. Larger tumours tended to show higher vascularity, and microvessels in larger tumours tended to show more dilation than those in smaller tumours (Fig. 3). These dilated blood vessels sometimes attained diameters of 200 µm.

The vWF antibody stained tumour microvessels in the malignant astrocytomas, but failed to stain microvessels in normal brain. Very little vWF immunoreactivity has been reported in capillaries of the normal brain [17], but in the present study, vWF immunoreactivity in such capillaries was lower than the sensitivity of our immunostaining method. Of 34 malignant astrocytomas, 24 tumours possessed vWF-positive or strongly positive microvessels, but 10 tumours did not. We analysed the relationship between tumour size and vWF immunoreactivity (Fig. 4). In all the tumours less than 1 mm in diameter, microvessels showed a vWF-negative reaction (Fig. 4A). In contrast, in all the tumours more than 2.5 mm in diameter, dilated microvessels showed a strongly positive vWF reaction (Fig. 4C). Hence, the malignant astrocytomas were classified into three groups: small tumours without vWF-positive tumour microvessels (8 tumours less than 1 mm in diameter), medium-sized tumours (11,

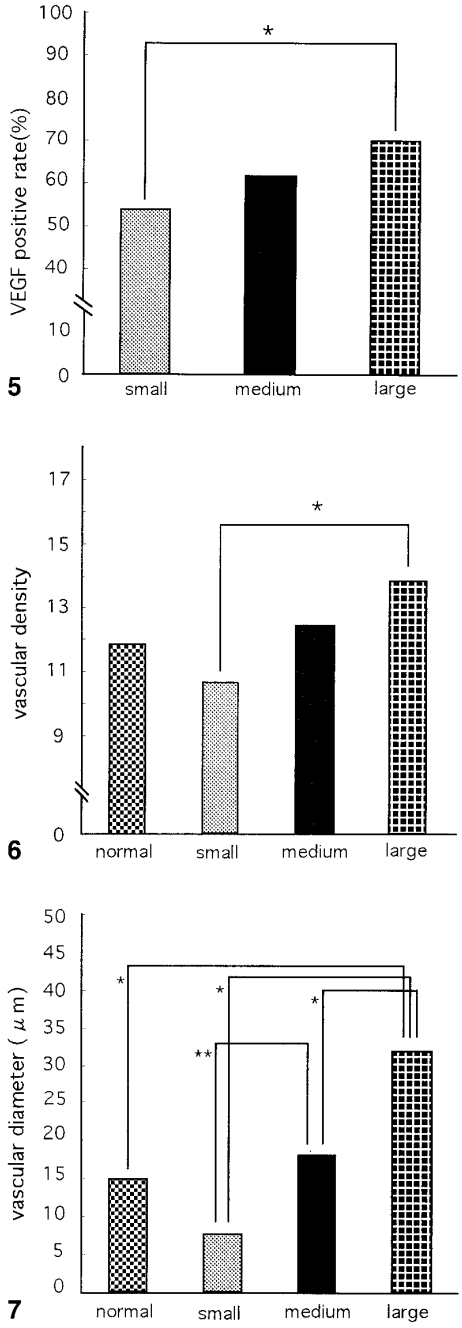
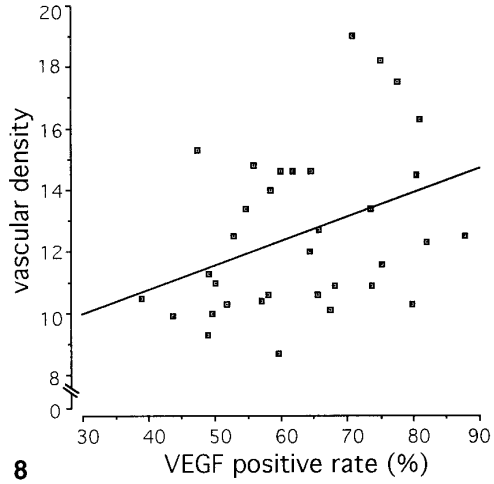


Fig. 5 Association of VEGF-positive rate with tumour groups. **P*<0.05 (post hoc Scheffé test). *small* tumours less than 1 mm in diameter; *medium* medium-sized tumours 1–2.5 mm in diameter; *large* tumours more than 2.5 mm in diameter

Fig. 6 Association of vascular density with tumour groups. **P*<0.05 (post hoc Scheffé test). *normal* normal brain tissues around the tumours

Fig. 7 Association of vascular diameter with tumour groups. **P*<0.001, ***P*<0.05 (post hoc Scheffé test)



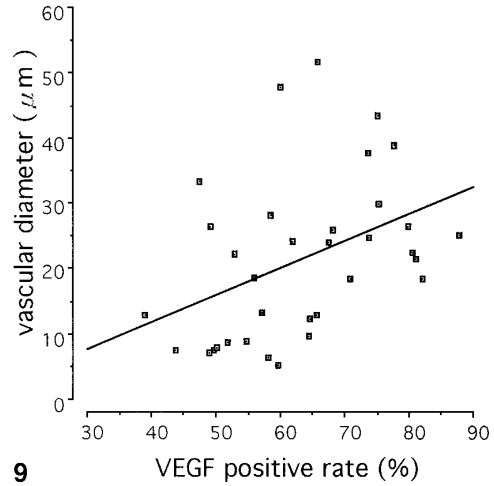


Fig. 8 Correlation of VEGF-positive rate and vascular density ($P<0.05$). *Continuous line* regression line

Fig. 9 Correlation of VEGF-positive rate and vascular diameter ($P<0.01$). *Continuous line* regression line

1–2.5 mm in diameter) with microvessels showing various vWF immunoreactivity, and large tumours with strongly vWF-positive tumour microvessels (15, more than 2.5 mm in diameter).

Comparisons were made of the VEGF(+)rate, vascular density, and vascular diameter between the three tumour groups. The mean value of the VEGF(+)rate was significantly higher ($P<0.05$) in the large (69.8%) than in the small (53.7%) tumours (Fig. 5), and the mean value of the vascular density was also significantly higher ($P<0.05$) in the large tumours (13.8) than in the small (10.4) tumours (Fig. 6). In addition, the mean value of the vascular diameter was significantly larger in the large (35.8 μm) than in the small tumours (8.7 μm) ($P<0.001$) (Fig. 7). Thus as tumours grew, their tissues showed higher VEGF(+)rates, higher vascular density, and larger vascular diameters. In the normal brain the mean value for vascular density was 11.0 and that for vascular diameter, 14.9 μm. The mean values for vascular density and vascular diameter were lower in the small tumours than in normal brain (not statistically significant).

In addition, we analysed the correlations between the VEGF(+)rate and the vascular density, and between the VEGF(+)rate and the vascular diameter, in order to clarify the role of VEGF in the induction of tumour microvessels. The increase in VEGF(+)rate correlated positively ($P<0.05$ and $P<0.01$, respectively) with an increase of vascular density (Fig. 8) and with an increase of vascular diameter (Fig. 9). VEGF expression may possibly have caused proliferation of the dilated tumour microvessels in the early-stage malignant astrocytomas.

Discussion

VEGF is believed to play an important part in angiogenesis of brain tumours. However, it is not clear whether early-stage brain tumours express VEGF. This study showed that the early stage rat malignant astrocytomas induced by ENU do express VEGF. All 34 malignant astrocytomas, even those less than 1 mm in diameter, showed positive immunoreaction against the VEGF antibody, suggesting that VEGF may have a role in the growth of the early-stage malignant astrocytomas. In addition, correlations between VEGF(+)rate and vascular density, and between tumour size and vascular density indicate that VEGF-induced angiogenesis may prompt the growth of early-stage rat malignant astrocytomas and of surgically removed human brain tumours, as reported previously [19, 20, 27].

A transparent chamber study has shown that the pre-existing vascular network is involved in growing tumours and gives rise to tumour vessels [11]. Support for this view is found in the present study. The microvasculature within the tumour varied with the size of the tumour. Small tumours (<1 mm in diameter) had microvessels without vWF immunoreactivity. In contrast, large tumours (>2.5 mm in diameter) always had microvessels with strong vWF immunoreactivity and dilatation. The medium-sized tumours (1–2.5 mm in diameter) appeared to be a transitional state in tumour vessel formation, their microvessels showing various intensities of vWF immunoreactivity and less dilatation. Similar changes in microvasculature have been found in SEM studies of vascular corrosion casts of ENU-induced rat brain tumours [14]. Brain tumours less than 2 mm in diameter showed hypovascularity and no tumour vessel formation, but those more than 2 mm in diameter had characteristic angiogenic microvessels and those more than 5 mm in diameter possessed strikingly dilated and deformed capillaries. Our statistical analysis indicated that VEGF expression may have caused proliferation of the dilated tumour microvessels in the large tumours. Microvessels in the small tumours probably consist of pre-existing microvessels, then, and when the ENU-induced brain tumours attain a diameter of about 1 mm microvessels affected by VEGF possibly initiate angiogenesis and the

formation of microvessels that increase the blood supply to proliferating tumour cells.

The vascular density of the small tumours tended to be lower than that of the normal brain, an hypovascular condition that may induce VEGF expression. Hypoxia and glucose deprivation induce VEGF expression, to satisfy tissue needs by expanding the vasculature [26].

Although nearly half the tumour cells showed a positive VEGF reaction, the small tumours still had no conspicuous angiogenesis, but in the large tumours, with an increase of the vascular density about 70% of the tumour cells showed a positive VEGF reaction. It seems likely that initiation of the angiogenesis may require accumulation of the VEGF-producing cells. It has been reported that there are correlations between VEGF immunopositivity and VEGF concentration and between VEGF concentration and microvascular density in glioma tissues [27]. Accumulation of the VEGF-producing cells may increase VEGF concentration in the tumour tissue to a sufficient level to initiate angiogenesis.

In the ENU-induced malignant astrocytomas, VEGF-positive reaction was observed in the tumour cells, vasculature, and reactive glial cells. The tumour cells were the majority of the VEGF-positive cells. VEGF immunostaining patterns vary in different reports, some immunohistochemical studies showing strong immunostaining predominantly in the microvasculature of gliomas as VEGF-binding sites [20–22] and another predominantly in the glioma cells [27]. This discrepancy is explained by the difference in the antibody used, as the antibodies recognize specific isoforms of the VEGF family [27]. Further studies are necessary to increase our understanding of the behaviour of each VEGF isoform in early tumours.

Reactive glial cells were a minor component of the VEGF-positive cells in the early-stage brain tumours. Since normal rat astrocytes express VEGF under hypoxic culture conditions [12], the hypoxia that probably occurs during early tumour growth may induce VEGF in reactive astrocytes as well as in tumour cells. Although VEGF-positive reactive glial cells were much fewer in number than were VEGF-positive tumour cells, they may have a particular role in early tumours.

VEGF, also known as vascular permeability factor, has the ability to increase the permeability of microvessels, primarily postcapillary venules and small veins, to circulating macromolecules [6]. It has been reported that the ENU-induced rat brain tumour showed a breakdown of the blood–brain barrier [5, 18]. The increase of vascular permeability in this brain tumour was seen in lesions more than 4 mm in diameter with a rich vasculature [13]. This stage may correspond to the formation of the tumour microvessels with a strongly vWF-positive immunoreaction and dilatation in the large tumours observed in this study. It seems, therefore, that VEGF may induce formation of hyperpermeable tumour microvessels that cause breakdown of the blood–brain barrier in the ENU-induced rat brain tumours.

vWF is a useful marker for brain tumour microvessels, because its synthesis is increased in endothelial cells of malignant brain tumours [17]. The present study indicates that VEGF possibly initiated formation of vWF-positive tumour microvessels. This view is supported by the pathophysiological study of human brain tumours by Goldman et al. [10], which suggests VEGF induced vWF release from endothelial cells. This study also suggests that vWF release may account for hypercoagulability manifesting as focal tumour necrosis. Since all the brain tumours more than 2.5 mm in diameter showed strong vWF-positive reaction, it seems that the microcirculation in such brain tumours may be affected by vWF. ENU-induced rat brain tumours often had necrotic areas [3, 18].

This study shows that VEGF may be an important angiogenic factor in early-stage brain tumours. Accumulation of the VEGF-positive tumour cells may lead to the initiation of angiogenesis and the formation of tumour microvessels, promoting the growth of the early-stage tumours.

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