LABORATORY INVESTIGATION

Histopathological vascular investigation of the peritumoral brain zone of glioblastomas

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Abstract
To date, no histopathological vascular investigation focusing on peritumoral brain zone (PBZ) has been reported for glioblastoma. We analyzed 10 newly diagnosed cases of glioblastomas. For these PBZs, histopathological investigation was performed by hematoxylin–eosin (H&E) staining and immunohistochemistry was analyzed for CD31, CD34, Factor VIII, VEGF, VEGFR-1/2, Ki67, p53 and nestin. Although it was difficult to identify PBZ by H&E, Ki67 and p53 staining, there were apparent differences in nestin staining among PBZ, tumor core (TC), and normal zone (NZ). Therefore, in this study, we divided PBZ from TC and NZ by nestin staining. Differences in histological features, microvessel density, expression of VEGF and its receptors were assessed for PBZ, TC and NZ. The microvessel density, as determined by counting CD31, CD34 and VEGF receptors, and VEGF-A expression were lower in PBZ than TC. The expression patterns for CD31, CD34 and VEGF receptors in vessels show dissociation in PBZ. In addition, the vascular characteristics of the PBZ may correlate with findings of radiographic imaging. We provide the first clinicopathological evidence that PBZ exhibits unique angiogenic characteristics. These in situ observations will help to elucidate the mechanisms of tumor recurrence.

Keywords Peritumoral · VEGF-A · VEGFR · CD34 · Nestin

Introduction
Glioblastoma is one of the most aggressive malignant brain tumors in humans, and is characterized by rapid proliferation and invasion into surrounding nervous tissues. A greater percentage of tumor resection, including the removal of the tumor invaded area, correlates with better prognosis. In general, tumor core (TC) is the area histopathologically occupied by tumor cells. Peritumoral brain zone (PBZ) is the invaded area with infiltrating tumor cells between TC and normal zone (NZ), that is mixed tumor and normal cells [1, 2]. Fluid-attenuated inversion recovery (FLAIR) high lesions of magnetic resonance imaging (MRI) have been found to be associated with PBZ in glioblastomas [3]. The resection of high intensity area on FLAIR is important, because this area represents the origin of recurrence after surgical resection of tumor [2]. However, gross total resection including that of high intensity area on FLAIR without the loss of neurological function is difficult for brain tumors [4]. Therefore, tools such as fluorescence-guided surgery with 5-aminolevulinic acid and navigation systems are used to aid in the resection of glioblastomas [5]. Although macroscopic and radiographic total resection can be achieved, almost all cases result in recurrence because of difficulties in clearly identifying PBZ using assistive tools during surgery [6].

Vascular endothelial growth factor (VEGF)-A is the important factor for the growth of glioblastoma [7]. VEGF-A binds to both VEGF receptor (VEGFR)-1 and VEGFR-2, promoting angiogenesis and vascular permeability [8–10]. The comparative analysis of differences in the angiogenic factor expression between grades of malignant gliomas has been previously reported [7]. The degree of VEGF-A expression in gliomas correlates with the grade of malignancy and prognosis [11]. In addition, it is also possible to predict the biological behavior of astrocytic tumors by observing the VEGFR-1 staining pattern [12]. However, angiogenic characteristics focused on PBZ have never been investigated. The
analysis of PBZ is necessary to understand the characteristics of glioma invasion in relation to angiogenesis.

We histopathologically examined how tumor vessels invade toward normal brain parenchyma and developed methods to clearly distinguish PBZ from normal brain parenchyma. In addition, the correlation between PBZ and the findings of radiographic images is also described in this report.

Materials and methods

Histopathological analysis

This research was approved by the Institutional Review Board at our institutes (Reference number: 20050002). Histopathological analyses were performed on 4-µm sections of formalin-fixed, paraffin-embedded tissue of ten tumors from ten patients with newly diagnosed glioblastoma.

Histological characteristics were assessed using hematoxylin and eosin (H&E) staining. Tumor proliferative activity was evaluated using immunohistochemistry with anti-Ki67 antibody (1:200, M7249, DAKO) as described previously [13]. The p53 protein is encoded by the tumor suppressor gene TP53, which is involved in regulation of the cell cycle, apoptosis, cell differentiation. Mutation in this gene is common and crucial in glioblastomas [14, 15]. The expression of p53 was examined using immunohistochemistry with an anti-p53 antibody (1:100, DO-7, DAKO). CD31 and CD34 can be shown in mature and immature endothelial cells [16]. The expressions of CD31 and CD34 were examined using immunohistochemistry with an anti-CD34 antibody (1:100, QBEnd 10; Dako) and anti-CD31 antibody (1:25, JC/70A, DAKO). Factor VIII can be observed in mature endothelial cells [17]. The expression of Factor VIII was examined using immunohistochemistry with an anti-Factor VIII antibody (1:25, M0616, DAKO). The expression of VEGF-A, VEGFR-1 (flt-1), and VEGFR-2 (KDR/flk-1) was examined using immunohistochemistry with anti-VEGF-A antibody (1:200, JH121, Merick Millipore), anti-VEGFR-1 antibody (1:100, AF321, R&D Systems), and anti-VEGFR-2 antibody (1:600, 55B11, Cell Signaling) [18, 19]. Nestin was first described as a neural stem/progenitor cell marker and is also considered as a marker of endothelial progenitor cells [19, 20]. The expression of nestin was examined using immunohistochemistry with an anti-nestin antibody (1:100, 10C2, Chemicon).

In immunohistochemical analyses, antigen retrieval was performed in citrate buffer (pH 6 for CD31, Factor VIII, VEGFR-1, p53, and nestin), or in Tris buffer (pH 9 for CD34, VEGF-A, and VEGFR-2) using microwave irradiation, and the products were visualized with peroxidase-diaminobenzidine reaction.

The specificity of immunohistochemistry was checked using negative and positive controls. For negative controls, paraffin sections were incubated with non-immune mouse, rabbit, and goat IgG at the same concentration used for each antibody (Fig. 2d). Sections from other glioblastomas not used in this study were used as positive controls for each antibody.

Histopathologically, PBZ is defined as the transitional border zone between TC and NZ, that is mixed tumor and normal cells [1, 2]. First, the sections containing all three regions—TC, PBZ, and NZ—were selected by H&E staining. Then, we attempted to identify the PBZ using endothelial cell markers (CD31, CD34 and Factor VIII), VEGF-A/VEGFRs expression, and immunostainings of nestin, Ki67 and p53. There were apparent differences of nestin staining among TC, PBZ and NZ, however it was difficult to clearly distinguish by other immunostainings (Fig. 1). Therefore, in this study, further analysis was estimated for the area of TC, PBZ and NZ defined by the gradient of nestin staining.

For the assessment of microvessel density (MVD), the tissue sections were screened using both CD31 and CD34 immunohistochemistry in low-power fields (×40), and the five most vascularized regions (hot spots) were selected for each region. The counting of microvessels was performed on these regions at high power (HPF: ×200, 0.95 mm²) [18, 19]. The expression of VEGF-A and VEGFR-1/2 was assessed by the consensus of three authors (RT, KO, and MT). The ratio of MVD in PBZ to TC assessed by CD31 and CD34 staining was calculated as a means of the degree of angiogenesis in PBZ (MVD PBZ/TC). Serial paraffin sections were immunostained and evaluated for each staining.

Radiographical analysis

Ten cases with newly diagnosed glioblastomas studied in this report showed ring-enhancing lesions. The FLAIR-high area around the gadolinium (Gd)-enhanced lesion and the Gd-enhanced area were measured for every slice by a picture archiving and communication system (PACS), and the combined areas were calculated by adding them together. The ratio of FLAIR-high area to Gd-enhanced area (FLAIR/Gd) was calculated as a means of the degree of FLAIR-high lesions.

Statistic analysis

Student’s t test was used to compare MVD, mitotic count, staining indices of MIB-1 for the TC, PBZ, and NZ of the ten tumors. The Pearson correlation coefficient was used to evaluate the linear relationship between MIB-1 index of PBZ, MVD PBZ/TC, and FLAIR-high area. Analyses were performed with IBM SPSS statistics.
Fig. 1 Comparable histopathological images of PBZ. Comparative analysis by each staining in Case 5 shows that PBZ is clearly distinguished by nestin staining, but not by other stainings. The borderline between TC and PBZ is designated by the red arrow, and the borderline between PBZ and NZ by the yellow arrow. (original magnification ×20, magnification bar: 100 μm). Tumor core (TC), peritumoral brain zone (PBZ): the invaded area with infiltrating tumor cells between TC and NZ, that is mixed tumor and normal cells, normal zone (NZ)
Results

Histological characteristics (Table 1)

In TC, the tumor was mainly composed of medium-sized astrocytic cells with increased cellularity and abundant mitotic figures. Marked palisading necrosis was observed and microvascular proliferation was predominantly present around this necrosis. Cellularity in PBZ gradually decreased and was decreased to a larger extent in NZ compared to TC. Mitotic count was extremely decreased in PBZ, and no mitosis was observed in NZ (TC: 10.9/10HPF, PBZ: 1.36/10HPF, and NZ: 0/10HPF, p < 0.01 for TC versus PBZ, p = 0.002 for PBZ versus NZ). MIB-1 index also showed same tendency (TC: 31.7, PBZ: 15.4, NZ: 0, p = 0.015 for TC versus PBZ, p = 0.08 for PBZ versus NZ).

Vascular analysis

Mean MVD assessed by both CD31 and CD34 staining was significantly decreased in PBZ compared to TC in all cases (mean TC: 55.24/5HPF, PBZ: 29.18/5HPF, and NZ: 8.6/5HPF, p < 0.01 for TC versus PBZ, p < 0.01 for PBZ versus NZ). Mean MVD assessed by VEGFR-1 and -2 expression also showed the same tendency (VEGFR1, mean TC: 55.24/5HPF, PBZ: 10.25/5HPF, and NZ: 0.11/5HPF, p < 0.01 for TC versus PBZ, p < 0.01 for PBZ versus NZ; VEGFR-2, mean TC: 53.13/5HPF, PBZ: 13.7/5HPF, and NZ: 0.18/5HPF, p < 0.01 for TC versus PBZ, p < 0.01 for PBZ versus NZ).

In all of the 10 cases, VEGFR-1 expression was observed in the endothelial cells and a subset of tumor cells in TC. The expression of VEGFR-2 was observed only in vascular endothelial cells in TC. However, VEGFR-1 and -2 expression appeared to be weak to negative in PBZ. The expression of VEGFR-1 and -2 was not detected either in vascular endothelial cells or tumor cells in NZ. In TC, all CD31- and CD34-positive endothelial cells exhibited both VEGFR-1 and -2 expressions (Fig. 2a). However, few CD31- and CD34-positive endothelial cells showed VEGFR-1 and -2 expressions in PBZ (Fig. 2b). CD31- and CD34-positive endothelial cells exhibited almost no VEGFR-1 or -2 expressions in NZ (Fig. 2c). A dissociation was observed between CD31, CD34 and VEGFR-1/2 expressions in PBZ.

CD31- and CD34-positive endothelial cells without VEGFR-1 and -2 expressions in PBZ were positive for nestin but negative for Factor VIII, which suggests immature tumor vessels [17, 21, 22]. In contrast, CD31- and CD34-positive endothelial cells in TC were positive for both nestin and Factor VIII, which suggests large proliferating tumor vessels [17]. In NZ, almost all CD31- and CD34-positive endothelial cells were nestin negative and Factor VIII positive, which suggests mature vessels [17].

Nestin can label several cell populations in glioblastoma, such as glioma cells including tumor stem cells, reactive astrocytes, proliferating endothelial progenitor cells. However, nestin-positive endothelial cells are distinguished from nestin positive reactive astrocytes and glioma cells by their shape and location (Fig. 2e). Nestin-positive endothelial cells are observed along the lines of vessel lumen that expresses CD31 and CD34. Compared with glioma cells, large-sized reactive astrocytes have abundant eosinophilic cytoplasm that expresses nestin. Although it is not easy to distinguish small-sized reactive astrocytes from glioma cells, reactive astrocytes tend to show an absence of nuclear atypia and mitosis [23]. In TC, few reactive astrocytes could be identified, whereas in PBZ, a small number of reactive astrocytes were identified.

VEGF-A expression (Table 1)

The positivity of VEGF-A staining in tumor cytoplasm or stroma was assessed as the following: ++: diffuse intense staining; +: diffuse faint staining; and −: negative staining [19]. In all of the 10 cases, VEGF-A staining was diffuse intense (+++) in TC (Fig. 2a), diffuse faint in PBZ (Fig. 2b), and negative (−) in NZ (Fig. 2c).

The relationship between radiographical FLAIR-high area and histological PBZ

The relationship between radiographic FLAIR-high area and histopathological parameters in PBZ was evaluated. Cases with narrow FLAIR-high areas tended to show low MVD PBZ/TC, and low MIB-1 index of PBZ (Fig. 3a). In contrast, cases with broad FLAIR-high areas tended to show high MVD PBZ/TC, and high MIB-1 index of PBZ (Fig. 3b). There was a significant linear relationship between FLAIR-high area and histopathological characteristics; MVD PBZ/TC and MIB-1 index in PBZ (Fig. 4a, b).

Discussion

Glioblastomas are highly malignant tumors that exhibit extensive vascularity. VEGF-A is strongly expressed in glioblastomas, with the expression approximately 10 times higher than in grade II gliomas [24]. The expression of grade III gliomas was also extremely low compared to that of grade IV gliomas [25]. In all cases of this study, the expression of VEGF-A was lower in PBZ than in TC. However, the level of VEGF-A expression in PBZ of glioblastoma may be
Table 1 Results of immunohistochemical and radiographical analyses of the 10 newly diagnosed glioblastomas

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<th>VEGF-A</th>
<th>MVD (VEGFR1) (mm²)</th>
<th>MVD (VEGFR2) (mm²)</th>
<th>MVD PBZ/TC (%)</th>
<th>FLAIR (cm³)</th>
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VEGF-A was assessed as the following: ++: diffuse intense staining, +: diffuse faint staining, −: negative staining
MVD PBZ/TC: the ratio of MVD in PBZ to MVD in TC assessed by CD31 and CD34
FLAIR/Gd: the ratio of FLAIR-high area to Gd-enhanced area

higher than that in grade III gliomas shown in the previous study [24, 25]. VEGF-A is induced by autocrine process and HIF-1α under hypoxic conditions [26]. The cellularity was high in PBZ of glioblastoma, that might cause autocrine process and hypoxia, inducing VEGF-A expression. In contrast, NZ displayed no VEGF-A expression.

CD34 is a marker of vascular endothelial cells and hematopoietic stem cells. Asahara et al. demonstrated CD34-positive cells also expressed CD31, CD133, VEGFR-2, Tie2, and VE cadherin. During differentiation, endothelial progenitor cells begun to express VEGFR-1, Tie1 and E selectin [27, 28]. Therefore, the expression of CD31, CD34 and VEGFR-1/2 (especially VEGFR2) showed parallel in general. In addition, Huang et al. showed the expression of VEGF-A also corresponds to those of VEGFRs in glioblastomas [29]. MVDs counted by VEGFRs gradually decreased in PBZ compared to that in TC, consistent with the data of MVDs counted by CD31 and 34. However, PBZ exhibited a difference between the expression of CD31, CD34 and VEGFRs in our study. In TC, almost all CD31- and CD34-positive endothelial cells showed positive expression of VEGFR-1 and -2. In PBZ, most CD31- and CD34-positive endothelial cells did not express VEGFRs. Furthermore, NZ did not display VEGFRs expression at all. All CD31- and CD34- positive endothelial cells without VEGFR-1 and -2 expressions in PBZ show nestin-positive but Factor VIII-negative, which suggest immature tumor vessels. The VEGF-A staining is diffusely faint. (original magnification ×400, magnification bar: 100 μm).

As Figure 2A and B show, VEGF-A staining in TC is strongly and diffusely intense (++). There are numerous nestin-positive tumor cells and endothelial progenitor cells. During differentiation, endothelial progenitor cells began to express VEGFR-1, Tie1 and E selectin [27, 28]. Therefore, the expression of CD31, CD34 and VEGFR-1/2 (especially VEGFR2) showed parallel in general. In addition, Huang et al. showed the expression of VEGF-A also corresponds to those of VEGFRs in glioblastomas [29]. MVDs counted by VEGFRs gradually decreased in PBZ compared to that in TC, consistent with the data of MVDs counted by CD31 and 34. However, PBZ exhibited a difference between the expression of CD31, CD34 and VEGFRs in our study. In TC, almost all CD31- and CD34-positive endothelial cells showed positive expression of VEGFR-1 and -2. In PBZ, most CD31- and CD34-positive endothelial cells did not express VEGFRs. Furthermore, NZ did not display VEGFRs expression at all. All CD31- and CD34- positive endothelial cells without VEGFR-1 and -2 expressions in PBZ show nestin-positive, and Factor VIII-negative, which suggest immature tumor vessels. The VEGF-A staining is diffusely faint. (original magnification ×400, magnification bar: 100 μm).

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Fig. 3 Relationship between FLAIR-high area and PBZ.  

**a** MRI of Case 5 shows a narrow FLAIR-high area around Gd-enhanced lesion. PBZ is identified by nestin staining and shows low MVD PBZ/TC and MIB-1 index. (original magnification ×40, magnification bar: 100 μm).  

**b** MRI of Case 3 shows a broad FLAIR-high area around Gd-enhanced lesion. PBZ is identified by nestin staining and shows high MVD PBZ/TC and MIB-1 index. (original magnification ×40, magnification bar: 100 μm)

Fig. 4 Relationship between FLAIR-high area and MVD of PBZ. The degree of FLAIR-high areas correlates with MIB-1 index of PBZ (a), and the MVD PBZ/TC (b)
VIII-negative, which suggests immature vessels. This histopathological results demonstrate that immature endothelial progenitor cells first began to express only CD31, CD34 and nestin (not VEGFRs), and then they proliferate, expressing VEGFRs along the concentration gradient of VEGF-A [30, 31]. CD31-, CD34- and nestin-positive, but VEGFRs- and Factor VIII-negative vessels in PBZ may indicate the formation process of tumor vessels beginning to proliferate. The process of angiogenesis has the following four phases: (1) endothelial proliferation/differentiation; (2) sprouting (3) capillary formation and (4) intussusception [9, 32]. This dissociation between the expression of CD 31, CD34 and VEGFRs in PBZ implies the midpoint of this angiogenetic process.

A previous report showed that VEGFRs correlated well with astrocytic tumor malignancy, even better than VEGF content [12]. For this reason, VEGFR-targeting drugs such as cediranib (a pan-VEGF receptor tyrosine kinase inhibitor) is associated with encouraging radiographic response rates and 6-month progression-free survivals [33, 34]. In addition, another previous study demonstrated that cediranib induced improved perfusion and oxygenation in patients with glioblastomas [34]. Therefore, cediranib appears to be an attractive treatment. However, PBZ cannot be treated by this type of drug, because tumor and endothelial cells did not highly express VEGFRs.

If tumor located on the noneloquent area is expected to be totally resected, distinguishing PBZ from TC and NZ is crucial because postoperative histopathological identification of NZ in the specimen indicates that surgical resection was sufficient. However, it was difficult to distinguish PBZ clearly by endothelial cell markers (CD31, CD34 and Factor VIII), VEGF-A/VEGFRs expression, and immunostainings of Ki67 and p53 (Fig. 1). In our study, PBZ could be distinguished clearly by nestin staining (Fig. 1). Nestin is an intermediate filament protein that is known as a neural stem/progenitor cell marker. Additionally, nestin is also expressed in proliferating endothelial progenitor cells [35]. In an orthotopic glioblastoma model, nestin is not expressed in the NZ [36]. In our study, the borderline between PBZ and NZ was clearly delineated by nestin staining. Postoperative specimens should be stained with nestin to identify NZ and to determine whether resection has been sufficient.

The resection of FLAIR-high lesions is important to improve postoperative outcome, because these lesions correlate with PBZ in glioblastomas [3]. PBZ is occupied by infiltrating tumor cells and normal cells [1, 2]. There are various types of tumors that have broad or narrow FLAIR-high areas around Gd-enhanced regions (Fig. 3a, b). Our results showed that MIB-1 index of PBZ correlated with the degree of FLAIR-high area (Fig. 4a). Low MIB-1 index of PBZ correlated with narrow FLAIR-high areas, and high MIB-1 index of PBZ correlated with broad FLAIR-high areas. In addition, the MVD ratio of PBZ to TC also correlated with the degree of FLAIR-high area (Fig. 4b). As described above, PBZ was occupied by immature, hyperlucent vessels, and therefore it may cause vasogenic edema and strong FLAIR-high areas. Bevacizumab (a recombinant, humanized monoclonal antibody against VEGF-A) can dramatically decrease FLAIR high lesion for patients with glioblastoma, due to normalization of the vascular structure and decrease of microvessel density [19, 37]. Therefore, vascular permeability of immature vessel in PBZ may influence FLAIR abnormality.

PBZ has different vascular characteristics compared to TC and NZ. These immature vessels in PBZ are suggested to be resistant to treatment with VEGFR-targeting drugs. The characteristics of PBZ may be related to the course of tumor progression and the findings of the radiographic images. Our findings provide further evidence for the reasons behind the recurrence of glioblastomas.

Conclusion

In glioblastoma, PBZ contained immature vessels that have different vascular characteristics, and can be clearly distinguished from TC and NZ by nestin staining.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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