Angiogenesis in glioblastoma — analysis of intensity and relations to chosen clinical data

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Microvascular proliferation (MVP) is one of the histopathological hallmarks of glioblastoma (GB). In this study angiogenic potential in GB was analysed according to the morphology of MVP and by assessment of vascular density. The analysis of relations of vascular parameters to chosen clinical features was performed. Tissue samples from 46 GB cases were examined. The clinical data included: patients’ age (32–78 years), gender (17 women, 29 men), location (frontal lobe – 13, temporal – 18, parietal – 14, two lobes – 1) and tumour size in CT (2–9 cm). Tumour vascularisation was analysed morphologically and quantitatively. Histologically two types of MVP were distinguished: simple and glomeruloid. In vascular hot spots vessel density was assessed on sections immunostained for vWF.

Simple type proliferation was found in all cases. Glomeruloid proliferation was found in 33 cases with mean age of patients 59.5 yrs, while in the group without glomeruloids mean age was 48 yrs (statistically significant difference, p < 0.01). Mean vascular density value in examined GB was 150.4 vessels/mm² (median 141.5; SD — 56.4) and younger age was related to higher vascular density (correlation coefficient R = −0.35; p = 0.017).

Vascular parameters were related only to the patients’ age among the analysed clinical data. The presented results show that morphologically microvascular proliferation is more intense in older GB patients, since higher vascular density is related to younger age. This observation may suggest the diversity of GB angiogenic potential depending on patients’ age.

key words: glioblastoma, angiogenesis, microvascular proliferation, vascular density

INTRODUCTION

Glioblastoma (GB) is the most malignant astrocytoma and the most common neuroepithelial neoplasm of the brain in adults. It grows extremely aggressively locally within the central nervous system (CNS) but very rarely raises systemic metastases [14]. Characteristic histological features of this tumour include cellular proliferation and pleomorphism, necrosis and a microvascular proliferation [5, 14, 15]. Necrosis in GB develops in areas where metabolic demands exceed oxygen supply or it arises from vascular thrombosis [14, 15, 19, 23]. Hypoxia can be a cause as well as a consequence of necrosis, leading to upregulation of expression of angiogenic factors, releasing them from dying cells or selected highly malignant cellular clones [19]. The vascular stroma of GB consists of many forms of blood vessels. There are vessels with usual structure — incorporated and formed de novo, and pathological vessels with
proliferative and degenerative changes — glomeruloid, telangiectases, haemangiomalike and sinusoidal structures [10, 13, 21, 28, 29]. In GB florid neovascularisation causes proliferation that exceeds the migration, remodelling and maturation of new vessels [23, 29]. Blood supply is necessary for tumour growth and metastasis, since it plays the nutritive role for the neoplastic tissue and provides the way of dissemination [4, 9, 26, 27]. Angiogenesis is a complex process regulated by growth factors and inhibitors released from neoplastic cells, endothelial cells and macrophages. The most important angiogenic factors in GB are: VEGF (Vascular Endothelial Growth Factor) with its receptors Flk1 and Flt1, angiopoietin 1 and 2 with their receptors, PDGF B (Platelet Derived Growth Factor B), and b FGF (basic Fibroblast Growth Factor) [10, 23, 31]. The intensity of intratumoral angiogenesis has become one of the markers of biological aggressiveness and a potential prognostic factor for patients with many types of cancer [4, 8, 9, 27, 31]. Research in this area has important implications resulting in antiangiogenic and angioregressive therapeutic regimens, which are being intensively evaluated in experimental and clinical trials [3, 4, 7, 31].

Brem et al. were the first authors to create an angiogenesis grading system based on vessel density, endothelial cell number and their cytological features [3]. They found GB as the most vascularised human neoplasm. The widely accepted method of estimating the extent of angiogenesis is nowadays the assessment of the microvascular density [8, 26, 27]. Numerous studies performed on different kinds of malignant solid tumours have proved the association of high vascular density with greater risk of metastases [8, 9, 26] and/or shorter patient survival. Some studies have not confirmed these results or showed inverse correlation [8, 9, 27]. It seems that the prognostic value of vascular density depends on the kind of neoplasm [7, 27, 28]. Moreover, this parameter gives only information about the presence of vessels, without consideration of their morphological, biological or functional properties [9, 17, 28].

The prognosis of the GB patients is very bad despite modern combined therapy possibilities [14, 15, 24]. Known established prognostic factors in this tumour are: preoperative Karnofsky performance status, extent of tumour resection and the patient’s age [6, 14, 24]. Antiangiogenic therapy represents a promising new therapeutic (target) modality in GB [1, 10, 31].

In the present study the angiogenic potential in GB was analysed according to the morphology of microvascular proliferation and by assessment of the vascular density. Moreover, correlations between vascular parameters and basic clinical data were examined.

**MATERIALS AND METHODS**

The examined material consisted of 46 cases of classic glioblastoma from patients operated on in the period 1995–1998 at the Neurosurgery Department of the Medical University of Gdańsk. The histopathological diagnosis was performed at the Pathology Department according to WHO 2000 CNS Tumours Classification [14].

The formalin-fixed, paraffin-embedded biopsy specimens of the tumours were cut into slides 5 μm thick and stained H-E. Histologically two types of the intensity of microvascular proliferation were distinguished: simple and glomeruloid type. Simple type proliferation was defined as hyperplasia of vascular wall cells within a vessel with single lumen. Glomeruloid proliferation was defined as hyperplasia and hypertrophy of cells within the vascular wall with multiple lumina. This type of microvascular proliferation was described as: slight (+) — scattered glomeruloids, prominent (+++) — multiple or grouped glomeruloids or intense (++++) with vascular conglomerations.

The most representative sections with the highest vascular condensation and without diffuse necrosis were selected for the immunohistochemical procedures with monoclonal antibody against vWF (vWF) (F8|86, 1:50). The sections were incubated with primary antibody at a temperature of 4°C for 20 hours. The designation of antigen was performed using the biotinylated secondary antibody and streptavidine conjugated to peroxidase (method LSAB 2: Dako), diaminobenzidine (DAB) was a chromogen. The mouse serum from the nonimmunised animal was used as a negative control instead of the primary antibody.

Quantitative analysis was performed with computed image analysing system (microscope Olympus BX50, camera CCD-FS-2012P — Bischke, software MultiScan v.5.10 — CSS). Tumour fields with the highest neovascularisation were identified at 40 bx. Under 100 × magnification 10 fields from these areas were recorded in the computer memory (one field — 0.31 mm²). Any immunopositive structure (round, oval ring or an irregular one) that was clearly separated from adjacent profiles and other tissue elements was considered a single countable vessel [27]. In the analysis of complicated structures, such as glomeruloids, vascular conglomerations and haemangiomalike forms, every separate lumen surrounded with chromogen was counted as a single vessel. The number of vessels was counted and the mean vessel number in every 10 fields per examined area was taken as the vascular density.

All calculated results were collected in a database for statistical evaluation. The mean value and standard deviation of age and vascular density were computed. Two-sided Student’s t-test was used to assess the level...
of significance of the differences between age and vascular density values in the group with different MVP and gender. The Pearson correlation coefficient was computed between the vessel density values and patients' age, vessel density and tumour diameter. A p-value $P < 0.05$ was considered statistically significant. Correlation status between MVP, localisation and gender was examined with the use of chi-square test ($p < 0.01$). All calculations were performed using The Statistica for Windows (v 5.1) program (StatSoft Inc. Tulsa, Ok, USA).

RESULTS

The patients were: 17 women and 29 men aged 32 to 78 years (mean 56.3 yrs, median 57 yrs). Women's mean age was 52.8 years and men's was 58.3 years. Tumour location was: frontal lobe (13), temporal (18), parietal (14), two lobes (1). Maximal tumour diameter measured in CT or MRI scans ranged from 2 to 9 cm.

The distribution of blood vessels and angioarchitecture within the tumours were heterogeneous. There were vascular-rich areas with plenty of small capillaries (Fig. 1), areas with microvascular proliferation — especially around the necrosis and also almost avascular fields. Microvascular proliferation of simple type was present in all of the cases together with the other types of microvessels. It was encountered focally or as multiple dispersed thickened microvessels (Fig. 2). Glomeruloid proliferation (Fig. 3) was found in 33 patients (Table 1). It was found in older patients, with the mean patients' age of 59.5 years and it was statistically significantly higher than in the group without glomeruloids — mean age 48 years; (Students t test $p < 0.01$) (Table 1; Fig. 4). Scattered glomeruloid vessels (+) were present in 7 patients, multiple (++) in 14 and conglomerations (+++) were present in 12 patients.

Vascular density values in examined cases ranged between 51.7 and 304.3 vessels/mm$^2$, with the mean value of 150.4 vessels/mm$^2$ (median 141.5, SD $-$ 56.4). The correlation was stated between patients' age and vascular density, Pearson correlation coefficient was $r = -0.35$; $P = 0.017$. The younger age was related to the higher vascular density (Fig. 5).

No statistically significant differences of the mean vascular density were stated between groups with different microvascular proliferation type. In the group with and without glomeruloid proliferation the mean vessel densities were 147.8 and 157 vessels/mm$^2$ respectively (Table 1).

No statistically significant relations were stated between vascular density or MVP type and tumour location, diameter and patients' gender.

![Figure 1. Vascular-rich tumour field with plenty of numerous microvessels (vWf, $\times$ 100).](image-url)
**Figure 2.** Microvascular proliferation of simple grade — vessels with thickened wall (HE, × 400).

**Figure 3.** Microvascular glomeruloid proliferation — multiluminal vascular tufts (HE, × 400).
GB is a paradoxical neoplasm with florid microvascular proliferation and necrosis as its histological hallmarks [5, 14, 15, 19]. Two distinct types of GB are distinguished based on their molecular genetic characteristics: primary developing de novo and secondary growing through progression from low grade astrocytoma [14, 15]. These basic GB types with diverse molecular characteristics occur in the patients of different ages — primary GB affects older patients [14, 15, 25].

The methods of assessment of intensity of tumour angiogenesis and its prognostic impact are still being elaborated. Since the article of Weidner et al. [27] it has been accepted that in tumour biopsy specimens the angiogenic capacity and intensity are reflected by the number of microvascular profiles in the area [8, 9, 26]. Fuller information about tumour neovascularisation is given however by the vascular diameter, perimeter, number of functional vessels and also chosen morphological features [7, 10, 21, 28]. The methods of assessment of the vascular density have still not been definitely standardised. In GB the criteria of counting the glomeruloid vessels are of basic importance. Two methods are in practice — every glomeruloid vessel is one vessel [17, 18, 28] or each lumen within the glomeruloid is counted separately [4, 12]. The first method can cause an underestimation of the truth value, moreover counting the vessels in random tumour fragments gives low values [18, 28, 29]. Density measured in hot spots, on small microscopic fields, gives higher results [8, 28, 29].

High vascular density in most types of tumours is an unfavourable prognostic factor mainly because it is related to the increased risk of neoplastic dissemination [4, 8, 27]. GB however very rarely gives metastases outside the CNS, so the question arises if GB is really an angiogenesis-dependent tumour [28]. Mean vascular density estimated in autopsy material on whole brain sections from patients with GB was found to be higher within the tumour than in the surrounding brain tissue, but in about 50% of examined tumour fields there was no difference with normal white matter [29]. In different brain tumours a microvascular count above 68–70 vessels per field was related to a worse prognosis [17, 18]. Abdulrauf et al. [1] observed a shorter survival time

### Table 1. Patients’ age and vessel density in groups with or without glomeruloid proliferation

<table>
<thead>
<tr>
<th>Glomeruloid proliferation</th>
<th>Number of cases</th>
<th>Age [years] mean (± SD)</th>
<th>Vascular density [number/mm²] mean (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent (-)</td>
<td>13</td>
<td>48 (± 8.4)</td>
<td>157 (± 66.1) NS*</td>
</tr>
<tr>
<td>Present (+) (+) (+++)</td>
<td>33</td>
<td>59.5 (± 11.5)</td>
<td>147.8 (± 53)</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>56.3 (±11.8)</td>
<td>150.4 (± 56.4)</td>
</tr>
</tbody>
</table>

* — two sided t-tests  
NS — statistically non significant

**DISCUSSION**

**Figure 4.** Patients’ age in groups with and without glomeruloid proliferation.

**Figure 5.** Correlation between patients’ age and vessel density.
and a greater risk of malignant progression of fibrillary astrocytoma with a microvessel count of more than 7. In their study microvessel density and CSF VEGF levels were also independent prognostic markers in low grade astrocytomas [1]. Wesseling et al. [28] did not find significant microvascular density differences between benign and anaplastic astrocytomas, suggesting that these tumours use pre-existing vessels. The authors postulated that, within GB, glomeruloid and classic angiogenesis in the form of a network of delicate microvessels occur only locally [28, 29].

The comparison of our own results with those of other authors is difficult due to different methodology and the way of presentation of results. The higher vascular density in the present study was found in younger age patients. In GB the younger patients’ age is a good prognostic factor, opposite to that of most extra CNS tumours, where the dynamics of cancer in younger patients is higher [5, 6, 15]. The dependence of angiogenesis intensity on patients’ age was stated also in one study of breast cancer patients [20]. The patients under 50 years had a significantly higher mean vessel count than the patients over 70 years, and no correlation with other historical parameters were found [20].

Experimental studies show that in older animals slow tumour growth is connected to more intense stromal reaction—fibrosis and less intense angiogenesis [16, 22]. Pili et al. [22] noted many small-size vessels in tumours in young adult animals, and in old animals the vessels were less numerous, with bigger diameters and thinned walls. There are suggestions that in older age angiogenesis is changed due to the different reactivity of vascular wall cells to growth factors — higher response of smooth muscle cells and lower reaction of endothelial cells [2].

The pathologically overexpressed angiogenesis in GB causes the capillary network to transform focally into glomeruloid vessels and vascular conglomerations [4, 19, 23]. These vessels are composed of an endothelial cell layer and mural proliferation of pericytes and/or smooth muscle cells [13, 14, 30]. Eberhard et al. [7] found a low microvessel pericyte coverage index in GB, but the authors did not consider microvascular proliferation. A low pericyte coverage index implies the functional immaturity of the tumour vascular bed and a changed potential for vascular remodelling [7].

The type of microvascular proliferation was considered in several studies as the measure of intensity of GB angiogenesis [12, 13, 17, 30]. The frequency of glomeruloid proliferations in our own material was 32/44, while in the others: 11/19 [30], 13/20 [28] and 32/38 [17] GB cases. Proliferation of this type in our study was more pronounced in older patients, together with lower vascular density. Furthermore, mean vascular density did not differ significantly between groups with and without glomeruloid microvascular proliferation. Such results in examined cases suggest a different angiogenic potential of GB within the age groups. Burger et al., [5] and Leon et al. [17] have also noticed a correlation between increasing proliferative changes and the age of GB patients. The above-mentioned vascular features of GB in older patients can in part explain their worse reaction to radiotherapy, due to low vascular perfusion rate and few functional vessels [6, 24].

The data according to the genetic type of the examined GB were not available, so it was impossible to analyse tumour angiogenic profiles in this aspect. The correlation of vascular parameters with patients’ age may suggest the connection of the angiogenic potential of GB to the tumour molecular profile. Recently the correlation between the loss of heterozygosity of chromosome 10 and the proliferation index and number of blood vessels in GB has been described [11]. However, in another study [25], neither PTEN nor TP53 gene mutations influenced microvessel density.

The presented results show that in GB some angiogenic features are age-related. Microvascular proliferative changes of glomeruloid type are more frequent in the older patients, and higher vascular density in the younger patients. Quantitative studies together with the morphological data give more information about the complex angiogenic capacity of GB.

REFERENCES