Endoglin Is a Better Marker than CD31 in Evaluation of Angiogenesis in Glioblastoma

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Aim
To compare endoglin (CD105) and the pan-endothelial marker CD31 in the assessment of angiogenesis in glioblastoma and to evaluate their values in the prognosis of this malignancy.

Methods
Forty-six cases of glioblastoma were included in this retrospective study. All cases were immuno-histochemically stained for endoglin (CD105), CD31, vascular endothelial growth factor (VEGF), and MIB-1 (Ki67). In order to assess microvessel density, positively stained microvessels were counted for each specimen in predominantly vascular areas (hot spot) at ×400 magnification. The intensity of VEGF staining was scored on a three-tiered scale. The proliferation index was expressed as a percentage of Ki67 positive cells.

Results
Median CD105 microvessel density (median 49 microvessels/field, range 27-99) was significantly higher than median CD31 microvessel density (median 37 microvessels/field, range 12-76). CD105 microvessel density was more closely correlated with VEGF (Spearman’s ρ = 0.421, P = 0.003) than with CD31 microvessel density (ρ = 0.330, P = 0.024). The proliferation index was significantly associated with CD105 microvessel density (Pearson’s r = 0.323, P = 0.028), whereas correlation could not be observed with CD31 microvessel density (r = 0.219, P = 0.142). Finally, patients with lower CD105 microvessel density had a longer survival than those with higher CD105 microvessel density (P = 0.045), whereas CD31 microvessel density had no influence on the survival time (P = 0.340).

Conclusion
CD105 is a more sensitive marker than CD31 in the evaluation of angiogenesis in glioblastoma. Our study is the first report of the better prognostic significance of angiogenesis evaluated with CD105 rather than with CD31 in glioblastoma.

Glioblastoma is the most common and most aggressive primary brain tumor. Even after careful surgical excision, radio and chemotherapy, this tumor shows a high percentage of recurrence and the mean survival is still very short, from several months to a year (1).

It is well known that angiogenesis is one of the most important factors in the progression of malignant tumors and that most malignant tumors develop their own vascular networks by secreting growth factors, such as vascular endothelial growth factor (VEGF), which stimulate endothelial migration and proliferation (2,3). Since malignant gliomas are highly vascularised, many efforts have focused on new therapy approaches based on the inhibition of angiogenesis.

Many studies over the last decade have examined the prognostic value of microvessel density in different types of malignant tumors. Most of these studies showed a positive correlation between microvessel density and tumor recurrence or survival (4-6), although some reported contrary findings, with no or negative correlation between these two parameters (7,8). A possible
reason for these discrepancies may be different methodologies used in the evaluation of microvessel density. In most reports, antibodies against the von Willebrand factor, anti CD31 or anti CD34 were used. These antibodies can visualize some newly formed blood vessels, but they can also react with normal blood vessels which are just trapped in the tumor tissue. Endoglin (CD105) is a homodimeric cell surface component of the transforming growth factor β (TGF-β) receptor complex (9). It is highly expressed on proliferating endothelial cells, but weakly or not at all expressed on normal vessels, and it has therefore been suggested as a marker of angiogenesis (10).

The aim of this study was to compare monoclonal antibody anti-CD31 (mAb), a pan-endothelial marker commonly used to determine microvessel density, with endoglin (anti-CD105) mAb. Moreover, the expression of VEGF and the proliferative activity (Ki67) of tumor cells was evaluated and their values correlated with microvessel density. Finally, in order to evaluate the possible prognostic significance of microvessel density, the vessel density obtained by either anti-CD31 mAb or anti-CD105 mAb were compared with the overall patients’ survival.

**Material and Methods**

Forty-six patients who underwent surgery of primary glioblastoma between January 1995 and December 2002 at the Clinical Medical Center of Rijeka were included in this retrospective study. Recurrent tumors or tumors with a history of previous low-grade astrocytoma were not analyzed. Surgical specimens were fixed in 10% buffered formalin and embedded in paraffin blocks. Tumors were histopathologically classified on routine hematoxylin and eosin sections as glioblastoma according to the World Health Organization (WHO) Classification of Brain Tumors (11). Serial 4-μm sections were prepared from each sample and served for staining and immunohistochemistry. For each sample, all sections were taken from the same paraffin block in order to evaluate identical areas for staining different antigens.

**Immunohistochemical Analysis**

A sensitive streptavidin-biotin staining method (LSAB, DAKO Cytomation Corporation, Carpinteria, CA, USA) was used to highlight endothelial cell/microvessel density, the proliferative rate, and VEGF on tumor cells. For the staining of endothelial cells, anti-CD31 monoclonal antibody (mAb, clone JC70A, DAKO) at 1:50 dilution and anti-CD105 (SN6h, DAKO) at 1:25 dilution were used. For assessing the proliferation of tumor cells, mAb MIB-1, corresponding to Ki67 cDNA fragment, was used at 1:50 dilution (DAKO). VEGF expression was determined by using mAb VEGF (C-1) at 1:100 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For endoglin immunostaining, predigestion with proteinase K enzyme (DAKO) was performed at room temperature for 10 minutes. Before staining, the sections for CD31, MIB-1 and VEGF were treated in four washes of 10 mmol/L citrate buffer in a microwave for 5 min.

Negative controls were obtained by substituting primary antibodies with non-immune serum.

**Assessment of Immunostaining**

Microvessel density assessed by immunostaining for CD31 and CD105 was determined according to Weidner (12). Most vascular areas (so called hot-spots) in the tumor were located at low magnification (×40) and then counted at ×400 magnification. Each positive endothelial cell or group of cells in contact with a spot was counted as an individual vessel. The mean vessel count from three fields was used as CD105 microvessel density or CD31 microvessel density.

MIB-1 nuclear staining was quantified on the Image Analysis System. For each case, 1,000 cells were counted and the percentage of stained cells was expressed as the Ki67 proliferation index.

VEGF immunostaining was evaluated by using a semi quantitative scale: score 1 – <25% of VEGF-positive tumor cells; score 2 – 25-50% of VEGF-positive tumor cells; score 3 – >50% of VEGF-positive tumor cells (13).

**Statistical Analysis**

Continuous variables were compared by Mann-Whitney U or Kruskal-Wallis tests. The Pearson correlation was used to assess the relationship between two continuous variables. The Spearman correlation was used to assess the correlation between categorical variables. Survival probabilities were computed according to Kaplan-Meier method. The log-rank test was used for univariate analyses of the overall survival. Overall
survival was defined from the day of the initial surgery until the death of the patient. Survival until the end of the observation was considered as censored. Data were analyzed by Statistica 6.1 software (StatSoft Inc., Tulsa, OK, USA).

Results

The median CD31 microvessel density was 37 microvessels/field (range, 12-76) compared with 49 (range, 27-99) for the CD105 microvessel density ($P<0.001$, Wilcoxon test). A statistically positive correlation was observed between microvessel density stained by both anti-CD31 mAb and anti-CD105 mAb ($r=0.645$, $P<0.001$) (Fig. 1).

Both CD31 and CD105 microvessel density were positively associated with the expression of VEGF, although CD105 microvessel density showed a stronger correlation than CD31 microvessel density (Spearman’s $r=0.421$, $P=0.003$ and $r=0.330$, $P=0.024$, respectively). The median CD105 microvessel density significantly increased with the increase in VEGF score ($P=0.039$, Kruskal-Wallis test), whereas this was not significant for CD31 microvessel density ($P=0.071$) (Table 1, Fig. 2).

Table 1. Expression of vascular endothelial growth factor (VEGF) and microvessel density in glioblastomas as determined with CD105 and CD31 monoclonal antibodies

<table>
<thead>
<tr>
<th>VEGF score</th>
<th>No. of patients</th>
<th>Microvessel density (median, range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>CD105: 38 (32-44), CD31: 34 (33-35)</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>CD105: 43 (27-80), CD31: 34 (14-55)</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>CD105: 62 (30-99), CD31: 44 (12-76)</td>
</tr>
</tbody>
</table>

*VEGF score: 1 – <25% of VEGF positive tumor cells; 2 – 25-50% of VEGF positive tumor cells; 3 – >50% of VEGF positive tumor cells.

Microvessel density was correlated with Ki67 proliferation index of tumor cells. There was an association between CD105 microvessel density and Ki67 index (Pearson’s $r=0.323$, $P=0.028$), but this was not the case with CD31 microvessel density ($r=0.219$, $P=0.142$) (Fig. 3).

Finally, microvessel density assessed in two different ways was correlated with patients’ survival. In univariate analysis, the survival was significantly longer (median survival 292 days, range, 1-1,054) in patients with lower microvessel counts (ie, decreased median CD105 microvessel density) in comparison with shorter survival time

![Figure 1. Correlation between microvessel density (MVD) in glioblastomas, determined by an anti-CD105 and anti-CD31 antibody (Pearson’s $r=0.645$, $P<0.001$).](image1)

![Figure 2. Higher expression of vascular endothelial growth factor (VEGF) in glioblastoma tumor cells is accompanied by more microvessels determined with anti CD105 antibody ($P=0.039$, Kruskal-Wallis test). Box-and-whisker plots of CD105 microvessel density (MVD), VEGF score: 1 – <25% of VEGF positive tumor cells; 2 – 25-50% of VEGF positive tumor cells; 3 – >50% of VEGF positive tumor cells.](image2)

![Figure 3. Association of CD105 microvessel density (CD105MVD) with Ki67 proliferation index of glioblastoma cells (Pearson’s $r=0.323$, $P=0.028$).](image3)
(median survival 135 days, range, 1-969) in patients with higher microvessel counts (ie increased median CD105 microvessel density) ($P=0.045$, log-rank test). This correlation was not found for CD31 microvessel density ($P=0.340$) (Fig. 4).

**Discussion**

Considering that prognosis in glioblastoma is poor and conventional therapeutic treatments are rather unsuccessful, there is a high interest in alternative therapies. Since high microvessel proliferation is a hallmark of glioblastoma and some authors have shown that microvessel density is a prognostic factor of astroglial tumors (6), anti-angiogenic therapy becomes very promising.

This study demonstrated the superiority of CD105 over CD31 as a marker of angiogenesis in glioblastoma. These results are in accordance with those reported for breast cancer, where it was also established that endoglin is a more sensitive marker for microvessel proliferation (14) and a better prognostic marker (15). Furthermore, in colorectal and endometrial carcinomas, more microvessels were determined by CD105 antibody than by pan-endothelial markers (16,17). Nevertheless, opposite results have also been obtained, with more vessels recognized by pan-endothelial markers than by CD105 antibody, probably because of preexisting vessels entrapped in the tumor and recognized by pan-endothelial marker (13). But even in this case, endoglin was a better prognostic marker than the pan-endothelial marker.

These results point to a problem frequently encountered in studies dealing with the assessment of angiogenesis. The estimation of revascularization requires an optimal methodological approach, first of all in choosing adequate antibodies for (activated) endothelial cells and, secondly for appropriately assessing the vessel density. Various immunohistochemical markers were used to estimate microvessel proliferation in glial tumors, such as pan-endothelial markers (factor VIII, CD31, CD34) or antibodies against angiogenic proteins like VEGF, TGF-$\beta$, epidermal growth factor (EGF), and platelet derived growth factor (PDGF). Antibodies to pan-endothelial markers such as FVIII and CD31 react well with endothelial cells of large vessels, but their expression is decreased and even absent in newly formed tumor vessels (18,19), whereas CD34 can also stain some mesenchymal cells. Consequently, the use of pan-endothelial cell markers facilitates the assessment of the vascular status of a tumor but does not give an indication of the angiogenic status. Therefore, using marker molecules that are upregulated during angiogenesis is more appropriate.

The results that obtained with endoglin are in accordance with the functional activity of the CD105 molecule as a marker of proliferating endothelial cells. In fact, CD105 antibody primarily marks activated endothelial cells which participate in the ongoing angiogenesis (20). It is a component of the TGF-$\beta$ receptor complex, a cytokine that participates in cellular proliferation, differenti-
expression, in our study confirms the above arguments. A significant correlation between VEGF expression and CD105 microvessel density was also found in other types of tumors (23). Furthermore, CD105 microvessel density correlated with the proliferation index, whereas CD31 microvessel density did not. This suggests that tumor angiogenesis could play a role in promoting cell proliferation, as in some other tumors (24).

Finally, this study showed that endoglin is a better prognostic marker than CD31, since the median survival times were significantly shorter in patients with greater CD105 microvessel density, but not with greater CD31 microvessel density.

To our knowledge, this is the first study demonstrating better prognostic value of CD105 antibody than CD31 antibody in glioblastoma. This observation implies that endoglin and TGF-β are potentially important targets in further developing antiangiogenic therapy.

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