ANGIOGENESIS is considered a prerequisite for the continued growth of solid tumor; therefore, antiangiogenic therapy might lead to inhibition of tumor growth. In 1972, a quantitative method for histological grading of tumor angiogenesis, the microscopic angiogenesis grading system (MAGS), was published. The MAGS score is based on three parameters: vasoproliferation, endothelial cell hyperplasia, and endothelial cytology. Glioblastomas multiforme showing glomeruloid florid microvascular proliferation, a well-known histological feature of malignant glioma, appeared to be on the endothelial-rich end of the spectrum. The high MAGS score in glioblastoma multiforme suggested that these endothelial-rich tumors depend more on extensive neovascularization for their continued growth than do endothelial-poor tumors; therefore, antiangiogenic drugs were considered a potential therapy for malignant glioma. However, because the MAGS score is assessed in an area of maximum vascular density, it is liable to interobserver variation and does not account for the heterogeneity of the tumor tissue. Furthermore, the well-vascularized appearance of malignant glioma might be misleading, because the surrounding normal brain might be supplied with delicate blood vessels that are not as easily seen as the prominent tumor vessels. In our study, a more objective method consisting of a quantitative analysis of the specifically visualized microvasculature in glial tissue is described and several vascular parameters in whole-tumor sections of untreated human glioblastoma multiforme are compared with those in histologically normal cerebral cortex and white matter.
Quantification of microvasculature in glioblastoma

TABLE 1

<table>
<thead>
<tr>
<th>Definitions of quantitative histological parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>vessel number</td>
</tr>
<tr>
<td>number of complete vascular profiles in a field; each vascular profile demarcated from the surrounding tissue by a continuous basement membrane was considered a separate blood vessel; a glomeruloid blood vessel with multiple lumina and surrounded by a continuous basement membrane was thus considered one vascular profile; vessel number is the vascular density of the field</td>
</tr>
<tr>
<td>vessel area</td>
</tr>
<tr>
<td>sum of areas (given in square microns) of all individual vascular profiles in a field</td>
</tr>
<tr>
<td>vessel perimeter</td>
</tr>
<tr>
<td>sum of perimeters (given in microns) of all individual vascular profiles in a field; vessel perimeter indicates the microvascular surface potentially available for diffusion of oxygen and nutrients in a field</td>
</tr>
<tr>
<td>vessel diameter</td>
</tr>
<tr>
<td>approximation of the mean diameter of all complete vascular profiles in a field; to assess this parameter, the square of the shortest distance of each pixel in a vascular profile to the edge of this profile is determined (distance expressed in number of pixels); then the mean of this value for all pixels in that vascular profile is assessed; finally, mean values for all complete vascular profiles in a field are averaged</td>
</tr>
<tr>
<td>tissue cellularity</td>
</tr>
<tr>
<td>number of nuclei outside the vascular profiles in a field, corrected for the area occupied by the vascular profiles in this field; tissue cellularity is expressed per 100 sq μ and indicates the cellularity of the extravascular tissue in a field</td>
</tr>
<tr>
<td>relative vessel perimeter</td>
</tr>
<tr>
<td>ratio between vessel perimeter and number of nuclei in the extravascular tissue in a field; the relative vessel perimeter indicates the microvascular surface available for diffusion per cell in the extravascular tissue in a field</td>
</tr>
</tbody>
</table>

Materials and Methods

The brains of seven adult humans with untreated cerebral glioblastoma multiforme were studied. These brains were fixed in formalin and cut in 1-cm-thick slices. One representative coronal or horizontal slice with tumor from each brain was embedded in paraffin. A sliding microtome was used to cut 12-μm whole-tumor sections containing the whole tumor and a variable but ample amount of histologically normal cerebral cortex and white matter. To highlight the vascular basement membranes, these sections were stained immunohistochemically with a prediluted mouse monoclonal anticollagen IV antibody and counterstained with periodic acid–Schiff (PAS) and hematoxylin staining. Immunochemistry for collagen IV was performed using the Autoprobe III (streptavidin-biotin)* while 3,3′-diaminobenzidine tetrahydrochloride was used as a chromogen.

For image acquisition, an image analysis system† was attached to a camera mounted on top of a light microscope. In every selected square, one microscopic field was digitized at ×100 magnification and shown on an image monitor. These images, measuring 0.17 sq mm each, were analyzed one by one. First, the observer set a threshold level in the normalized gray level to distinguish vascular profiles from surrounding tissue; this resulted in a binary image. Morphological procedures were used to remove noise and fill the lumina of the vascular profiles. The entire enclosed area of these profiles was used for the calculation of the vascular parameters. When necessary, comparison of the original and the binary image enabled correction of the binary image by erasing nonvascular structures and completing vascular profiles using a mouse. After acceptance of this binary image, a second threshold value was set to discriminate the nuclei in the tissue outside the vascular profiles. All calculated data were collected in a database for further statistical evaluation.

In each selected field, one observer (J.v.d.L.) assessed four vascular parameters (vessel number, vessel area, vessel perimeter, and vessel diameter), a parameter for the cellularity of the extravascular tissue (tissue cellularity), and one combined parameter (relative vessel perimeter). These quantitative histological parameters are defined in Table 1.

The mean value (mean), the median value (median), and the standard deviation of the six parameters were assessed for each case in all four tissue categories (A to D). Because the study was meant to compare tumor tissue (random and preselected) with normal cerebral cortex and white matter, the differences between the tissue types for the mean, median, and standard deviation of the parameters were statistically evaluated with a Wilcoxon matched-paired, signed-rank test, normal α = 0.05, with Bonferroni correction actual α = 0.017. To evaluate the reproducibility of this computerized quantitative analysis, the scores of two independent observers (P.W., J.v.d.L.) were compared for all parameters in 84 identical images of preselected tumor (B) and of normal cerebral white matter (C).

Results

Light Microscopy

The combined PAS-collagen IV staining procedure resulted in a brown staining of the basement mem-

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* Mouse monoclonal anticollagen IV antibody and Autoprobe III Universal Detection System supplied by Biomeda Corp., Foster City, California.

† Kontron Vidas® image analysis system supplied by Kontron GmbH, Eching, Germany.
branes of the blood vessels and a blue-purple staining of the nuclei. Although most of the analyzed random tumor-tissue fields were located in white matter, especially in Cases 2 and 7, up to 20% of these fields were located in the cerebral cortex. In the analyzed fields in tumor tissue and in normal cerebral cortex and white matter, there appeared to be no particular orientation of the microvessels and arteries and veins were found...
only sporadically. In the preselected tumor fields (Category B), glomeruloid microvascular proliferation was extensively present in Cases 2 and 6; locally present in Case 4; sporadically present in Cases 1, 3, and 5; and absent in Case 7. The estimated number of blood vessels in these preselected tumor fields was locally high in Cases 3, 5, and 7; and moderate in Cases 1, 2, 4, and 6.

**Quantitative Analysis**

The results of the quantitative analysis and the statistical evaluation of these results are shown in Fig. 1 a to f. Examples of the scores for the six parameters in different fields are shown in Fig. 2 a to h. Compared to normal cerebral white matter, the standard deviation in random tumor for the vascular parameters (vessel number, vessel area, vessel perimeter, and vessel diameter) was significantly increased. The mean and median for vessel area and vessel perimeter and the mean for vessel number were significantly higher in random tumor than in normal cerebral white matter; however, in most cases, the score for vessel number in more than 50% of the random tumor fields was in the range of scores for normal cerebral white matter. In normal cerebral cortex, the mean and median for vessel number, vessel area, and vessel perimeter were significantly higher, and the mean and median for vessel diameter were significantly lower than in normal cerebral white matter.

The mean, median, and standard deviations for tissue cellularity were significantly higher in random and preselected tumor than in normal cerebral cortex and white matter, whereas in normal cerebral white matter the mean and median for this parameter were significantly higher than in normal cerebral cortex. The mean and median for relative vessel perimeter in random and preselected tumor were not significantly different from those in normal cerebral white matter and were markedly lower than those in normal cerebral cortex. In the random tumor fields, no significant correlation was found between tissue cellularity and the four vascular parameters.
The preselected tumor fields of Cases 2 and 6 with extensive glomeruloid microvascular proliferation showed a relatively high vessel area and vessel diameter compared to the preselected tumor fields in the other cases. On the other hand, a relatively high vessel number was found in the preselected tumor fields of cases in which the number of blood vessels before quantitative analysis was estimated as locally high (Cases 3, 5, and 7).

The correlation coefficient between the scores of the two independent observers was higher than 0.9 for all parameters in the 84 fields of preselected tumor and normal cerebral white matter. Between the scores of these observers, no relevant systematic or incidental differences were found for any parameters in normal cerebral white matter or for the vessel area, vessel perimeter, tissue cellularity and relative vessel perimeter parameters in preselected tumor. The highest systematic and incidental differences between the two independent observers occurred in the vessel number and vessel diameter parameters in preselected tumor; the systematic differences for vessel number and vessel diameter were 16.5% and 11.3%, respectively, and the incidental difference for both these parameters was 18.4% (p < 0.001). However, even these differences had little impact on the final scores for the mean, median, and standard deviation of vessel number and vessel diameter in preselected tumor.

Discussion

Quantitative Histological Studies on the Glioma Microvasculature

Few quantitative histological studies on the glioma microvasculature have been performed. A morphometric study of the microvasculature in and around rat glioma showed no significant rise in vascular density around the tumor and a drop of vascular density toward its center. In semithin sections of human glioblastoma multiforme, a significant decrease in vascular density was found in and around the tumor compared to normal white matter, although the vascular volume was increased in the tumor but decreased around the tumor. In a biopsy study of human cortex infiltrated by malignant glioma, an increase in vascular density only occurred in some cases of markedly and completely infiltrated cortex; this angiogenic reaction to tumor infiltration was considered as late, slow, and inconstant. In another study, a decrease in intercapillary distance was described in the proliferating area of human glioma. The heterogeneity of the microvasculature in glioblastoma multiforme has been illustrated by a morphometric study with a graphic tablet on microscopic photographs of these tumors.

Automated image analysis was recently introduced as a tool for a more objective histological assessment of angiogenesis in tumors and in this study, we describe a method for computer-assisted image analysis of the microvasculature specifically visualized in glial tissue. This method was applied to whole-tumor sections of glioblastoma multiforme that had not been treated so that microvascular status was not influenced by irradiation and/or chemotherapy. Furthermore, histologically normal cerebral cortex and white matter were present in all whole-tumor sections and could be used as internal controls.

Methodological Considerations

Accentuation of the objects of interest is of paramount importance for computer-assisted image analysis. Because in normal brain tissue and in glial neoplasms, collagen IV is essentially confined to the wall of blood vessels, the microvasculature in the whole-tumor sections was generally easy to identify in the combined PAS-collagen IV staining. In our study, a blood vessel was defined on the basis of a demarcation of the microvascular profile from the surrounding tissue by a continuous basement membrane, visualized in the PAS-collagen IV staining. In other studies, the number of vessel lumina in a field was determined as an indication for the vascularity of the tissue, and an immunohistochemical stain for endothelial cells was sometimes used to highlight those vessel lumina. In glioblastoma multiforme the number of microvascular lumina will generally be higher than the number of microvascular profiles as defined in this study, because some of the profiles contain multiple lumina.

In this report, the genesis of new microvessels in tumor tissue will be underestimated when a simultaneous expansion of the tumor tissue between the blood vessels occurs, such as by accumulation of tumor cells or by edema. However, because the vascular density in normal cerebral cortex is higher than in normal cerebral white matter, a relatively high number of pre-existing blood vessels can be expected in tumor tissue infiltrating cerebral gray matter. Increased vascular density without the occurrence of neovascularization might also be the result of collapse of the tumor tissue between the blood vessels, for example, by partial necrosis.

Florid microvascular proliferation in glioblastoma multiforme is often present around necrotic foci and in border zone areas in which neoplastic cells invade the adjacent brain. Unequivocal delineation of a tumor border zone can be difficult in glial neoplasms because of a diffuse infiltrative growth pattern; thus, we only performed the quantitative analysis in clear-cut tumor tissue and in normal cerebral cortex and white matter. The design of this study is thus not suitable to analyze the topographical distribution of microvascular proliferation in glioblastoma multiforme or to detect a relationship between this proliferation and necrosis in these tumors.

Unequivocal automated assessment of the diameter of branching vessels is difficult. Unlike vessel area and vessel perimeter, the vessel diameter parameter as defined in the present study is essentially not influ-
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Interpretation of the Present Findings

The results of the present study illustrate the striking heterogeneity of glioblastoma multiforme for all vascular parameters. Compared to normal cerebral white matter, the standard deviation in random tumor for vessel number, vessel area, vessel perimeter, and vessel diameter and the mean for vessel area, vessel perimeter, and vessel number were significantly increased, but the vessel number in more than 50% of the random tumor fields was in the range of the vessel number for normal cerebral white matter, suggesting that few new vessels had formed in such areas.

The results for vessel number and vessel diameter in the normal cerebral cortex illustrate the high number of delicate blood vessels in this tissue. The relatively high standard deviation for vessel number in the normal cerebral cortex might be explained by the variation of vascular density across the depth of the cortex. Although the mean and median for vessel perimeter in random tumor are significantly higher than in normal cerebral white matter, no significant difference is found for relative vessel perimeter between these tissues because of the relatively high cellularity of random tumor tissue.

Twelve fields of tumor tissue in each case were selected on the basis of the vascularity of the tissue. Because the criterion used for selection was not unequivocal (presence of glomeruloid microvascular proliferation and/or a relatively high number of blood vessels), a heterogeneous population of tumor fields was analyzed in this category of preselected tumor. The results of the analysis of these preselected tumor fields do, however, demonstrate the major effects of selection of fields on the basis of certain criteria before further quantitative analysis. The vessel diameter and vessel area were relatively high in preselected tumor fields with extensive glomeruloid microvascular proliferation, whereas the vessel number was relatively high in fields with an estimated high number of blood vessels. This phenomenon also illustrates that vascularity of tissue is a broad concept, not only determined by the vascular density, but also by other vascular parameters, such as vessel area, perimeter, and diameter. The presence of prominent blood vessels in glial tumor may give the impression of a highly vascularized tumor but does not necessarily mean that the vascular density is increased.

The reproducibility of this computerized method for quantitative analysis of the microvasculature is good. The only relevant differences between the two independent observers were found in vessel number and vessel diameter in preselected tumor fields. The very complicated microvascular pattern in some of these fields combined with a less crisp demarcation of some vascular profiles probably contributed to these differences. In most cases, however, the interobserver differences had little influence on the mean, median, and standard deviation of the parameters.

Pathobiological Significance of Microvascular Proliferation in Gliomas

More than 50 years ago, Scherer commented that astrocytic neoplasms generally show a diffuse, infiltrating rather than a solid growth pattern, the growth of the tumor cells being guided by pre-existent fiber tracts. It has been suggested that these tumors can spread using existing vessels rather than being supplied by new ones. The extent to which astrocytic neoplasms in general are angiogenesis dependent therefore remains to be established.

Theoretically, when the amount of tumor cells in a certain volume in astrocytic neoplasms exceeds a critical number and a more solid growth pattern occurs, or when tumor cells in a certain area become metabolically hyperactive (for example, in more malignant neoplasms), the tumor tissue may outstrip pre-existent blood vessels and become dependent on angiogenesis. This process is accompanied by local hypoxia, which is supposed to be an important factor in the development of microvascular proliferation in glioma. However, Russell and Rubenstein found that microvascular proliferation in glioblastoma multiforme is usually not accompanied by any regional increase in density of tumor cells. In the present study also no significant correlation was found between the cellularity of the tumor tissue (tissue cellularity) and the four vascular parameters (vessel number, vessel area, vessel perimeter, and vessel diameter).

Florid microvascular proliferation is a well-known feature of malignant glioma, especially of glioblastoma multiforme, and might be the result of production of angiogenic factors in tumor cells of glial neoplasms, combined with a lack of directional microvascular growth in the tumor tissue. In florid microvascular proliferation in glioma in situ, accumulation of both endothelial cells and vascular smooth-muscle cells or pericytes has been described. Because the contribution of these aberrant blood vessels to the viability of the tumor tissue is unclear, even in areas of malignant glioma with prominent microvascular proliferation, the efficiency of antiangiogenic therapy must be clarified.

Conclusions

The authors have described a feasible and reproducible computer-assisted image analysis method to quantify multiple parameters of the microvasculature in histological sections of normal and neoplastic glial tissue. Application of this method to whole-tumor sections of untreated human glioblastoma multiforme illustrates that the striking heterogeneity of the microvasculature in these tumors is such that the number of vessels in many tumor-containing areas does not exceed that of normal white matter. The relevance of these findings to the efficacy of antiangiogenic therapy remains to be defined. The results do suggest, however, that many intratumoral regions may not be overtly angiogenesis dependent or amendable to antiangiogenic therapy.
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