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MDR1 P-Glycoprotein Is Expressed by Endothelial Cells of Newly Formed Capillaries in Human Gliomas but Is Not Expressed in the Neovasculature of Other Primary Tumors

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The expression of human* MDR1 *P-glycoprotein (Pgp) in the capillary endothelial cells of the central nervous system has been demonstrated. The brain capillary endothelial cells maintain the structure and function of the blood-brain barrier. Recently, the human* MDR1 *Pgp (and its mouse homologue* MDR1a *Pgp) has been shown to function as an important part of this barrier, pumping out xenobiotics from endothelial cells into the lumen of capillaries resulting in the protection of the brain parenchyma. To examine whether the endothelial cells of the newly formed capillaries during neoangiogenesis within malignant human brain tumors express* MDR1 *Pgp, 35 adult surgical brain tumor specimens (29 gliomas and 6 tumors metastatic to the brain) were obtained from previously untreated patients and studied by a new immunohistochemical sandwich method developed in our laboratory using the JSB-1 monoclonal antibody. JSB-1 is specific for the Pgp product of the human* MDR1 *(and not* MDR3*) gene. This sensitive method allows the detection of Pgp in capillary endothelial cells of normal brain in conventional paraffin sections after formalin fixation. The endothelial cells of the newly formed capillaries in 25 of 29 gliomas (86%) and 3 of 6 metastatic tumors, immunostained positive for* MDR1 *Pgp. The tumor cells in 7 of 35 cases were also positive for Pgp. In the 35 brain tumor cases investigated, the endothelial cells were Pgp positive in the tumor-brain border and in the brain further from the tumor. Capillary endothelial cells of neovasculature in 137 malignant tumors (non-brain) obtained from previously untreated patients showed no* MDR1 *Pgp expression. These results demonstrated that* MDR1 *Pgp is expressed not only in the capillaries of normal brain but also in the majority of the newly formed capillaries of brain tumors. Multidrug resistance of brain tumors may result not only from the expression of resistance markers in neoplastic cells but also from the* MDR1 *Pgp expression in endothelial cells of tumor capillaries. Pgp in this special localization can exclude chemotherapeutic agents from tumor cells that are located around the capillaries. The therapeutic benefit and selectivity of chemotherapeutic agents in combination with a Pgp-reversing agent should be evaluated. (Am J Pathol 1996, 149:853–858)

The blood-brain barrier is located at the capillary endothelial-basal lamina-glial cell interface of the brain. Wide pores or fenestration are absent in these endothelial cells, unlike these cells elsewhere in the body. It has recently been shown that P-glycoprotein (Pgp), which is the product of the human multidrug resistance gene (*MDR1*) is highly expressed in human brain capillary endothelial cells,[1,2] and functional activity of Pgp in cultured cerebral capillary endothelial cells has been demonstrated.[3,4] Mammalian P-glycoproteins are encoded by two human (*MDR1* and *MDR3*) and three mouse (*mdr1a*, *mdr1b*,

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and *mdr2*) genes with various functions. Human *MDR1* and mouse *mdr1a* and *mdr1b* are responsible for multidrug resistance, but the closely related human *MDR3* and mouse *mdr2* do not appear to play a significant role in multidrug resistance (for review see Ref. 5).

Recently, there has been a breakthrough in understanding the role of Pgp function in the brain-blood barrier. Mice have been generated with disruption of the mouse *mdr1a* gene (homologous to human *MDR1*). Mice with the *mdr1a* knock-out have a deficiency in the blood-brain barrier and are more sensitive to Pgp substrate drugs such as vinblastine and the pesticide ivermectin.[5] This was the first direct evidence obtained from *in vivo* experiments suggesting that the mouse *mdr1a* Pgp (homologous to human *MDR1* Pgp) is a major functioning Pgp in the blood-brain barrier. Its absence resulted in elevated drug levels in brain.

The formation of new blood vessels including capillaries (tumor neoangiogenesis) in the stroma of the tumor is prominent in tumor development. Brain tumors are particularly dependent on angiogenesis. Previous reports have provided conflicting evidence (using frozen sections mostly) concerning whether *MDR1* Pgp is also present in the endothelial cells of the newly formed microvessels of human brain tumors. In two separate studies, no evidence of endothelial immunoreactivity for MRK16 (specific for *MDR1* Pgp) or C219 monoclonal antibodies (MAbs) was reported in a total of 32 glial tumors.[6,7] Other investigators, however, reported Pgp expression in endothelial cells of gliomas (six of eight cases) by immunohistochemical assay using MAb C219,[8] which recognizes both human *MDR1* Pgp and the irrelevant human *MDR3* Pgp. This latter result is, however, inconclusive because of possible cross-reactivity with human *MDR3* Pgp, which cannot transport drugs.[9] According to an earlier report, Pgp expression has also been detected in brain tumor capillaries on frozen tissue sections using MAb HYB-241 only,[10] but in this immunohistochemical study an isotype-matched antibody at the same concentration as the primary antibody was not included as a negative control and the publication does not provide information about the gene specificity of the monoclonal antibody used to detect the Pgp. These limitations do not allow a definitive conclusion about the presence of the *MDR1* gene product in endothelial cells of brain tumor capillaries.

We have developed a specific and sensitive immunohistochemical sandwich method for *MDR1* Pgp detection using MAb JSB-1.[11] Transfection studies have established that JSB-1 is specific for the Pgp product of the human *MDR1* (but not *MDR3*) gene.[9] We have demonstrated that this method allows reliable detection of Pgp in capillary endothelial cells of normal human brain capillaries in paraffin sections after formalin fixation.[11] Utilizing this method, we report here that *MDR1* Pgp is expressed not only in normal brain capillaries but also in the newly formed capillaries of the large majority of gliomas (25 of 29 cases), but not in 137 other tumors outside of the brain. We postulate that the presence of *MDR1* Pgp in endothelial cells of human brain tumor neovasculature represents a new role for Pgp in multidrug resistance.

Materials and Methods

Human Tumors

Conventionally processed paraffin sections (10% neutral buffered formalin fixation and paraffin embedding) of 35 adult surgical brain tumor specimens (29 gliomas and 6 tumors metastatic to the brain) and of 137 other malignant tumors (non-brain) obtained from patients with no prior therapy were analyzed. The majority of tumor specimens were received through the Tissue Procurement Facility of the Department of Pathology at Roswell Park Cancer Institute except for 18 brain tumors that were obtained from Neuropathology, Department of Pathology, State University of New York at Stony Brook. All gliomas were graded by a neuropathologist (N. S. Peress) starting with the most benign as grade I; numerical grades II, III, and IV represent increasing malignancy.[12,13]

Immunohistochemical Analyses

Assays were done using a new multilayer immunoperoxidase sandwich staining method developed in our laboratory for the specific and sensitive detection of human *MDR1* Pgp in formalin-fixed, paraffin-embedded sections.[11] Essentially, paraffin sections were sequentially exposed to the primary antibody JSB-1, followed by three additional layers of secondary antibodies. All of them were peroxidase labeled to amplify staining intensity (more enzyme molecules present per antigenic site). We have previously reported that capillary endothelial cells of normal brain showed strong positive immunostaining with JSB-1 but not with other MAbs.[11] Therefore, normal brain tissue was used as positive control. Negative controls included the isotype-matched antibody IgG1 at the same concentration as JSB-1 (1.4 μg/ml) and phosphate-buffered saline (PBS) alone in place of

Table 1. *Expression of* MDR1 *Pgp in Capillary Endothelial and in Tumor Cells of Human Untreated Brain Tumors and Other Primary Solid Tumors Evaluated by New Immunoperoxidase Sandwich Staining Method on Paraffin Sections*

Tumor type	Positive JSB-1 immunoreaction/cases		
	In capillary endothelial cells	In tumor cells	In both cells
Gliomas	25/29 (86%)	5/29 (17%)	3/29 (10%)
Metastatic tumors to brain	3/6	2/6	0/6
Primary breast carcinoma (unselected)	0/71 (0%)	23/71 (32%)	0/71 (0%)
Primary lung carcinoma (non-small cell)	0/26 (0%)	2/26 (8%)	0/26 (0%)
Primary prostate carcinoma (unselected)	0/10 (0%)	0/10 (0%)	0/10 (0%)
Miscellaneous tumors (carcinomas, sarcomas)	0/30 (0%)	6/30 (20%)	0/30 (0%)

the primary antibody JSB-1. Cells were considered (by K. Toth and N. S. Peress) positive for immunostaining when any or all of the following staining patterns were sufficiently intense to be clearly different from the appropriate isotype controls: plasma membrane staining or diffuse and/or granular (Golgi-pattern) cytoplasmic staining. A tumor sample was evaluated as negative when all endothelial or tumor cells lacked immunostaining. When the intensity of endothelial cell immunostaining in gliomas was as strong as in normal brain capillaries, the term strong was used. Weak was used when the staining was weaker in the tumor endothelial cells than was found in normal brain. The extent of JSB-1 immunoreactivity in endothelial cells was scored 1+ when less than 5%, 2+ when 5 to 75%, and 3+ when more than 75% of endothelial cells were immunostained.

As a positive control for comparison, immunohistochemical staining was also performed for the detection of factor-VIII-related antigen in selected cases (normal brain and four Pgp-positive and one Pgp-negative brain tumor). Anti-factor-VIII is a general marker commonly used for the detection of all vascular endothelial cells (small capillaries and larger vessels). Paraffin sections were incubated with a primary antibody against factor VIII, and antibody binding was visualized with a universal streptavidin/biotin staining kit (Lipshaw, Pittsburgh, PA). The staining procedure was performed according to the manufacturer's instructions.

Results

The data in Table 1 summarize the frequency of *MDR1* Pgp detection in capillary endothelial and tumor cells of untreated human brain tumors (primary and metastatic) as compared with other primary (non-brain) tumors studied by our multilayer immunohistochemical sandwich staining technique using MAb JSB-1 on paraffin sections. In 25 of 29 gliomas and 3 of 6 metastatic tumors, the endothelial cells of the newly formed capillaries immunostained positive for *MDR1* Pgp. In contrast, in 137 other (non-brain) malignant tumors, capillary endothelial cells of the neovasculature showed no JSB-1 immunostaining, indicating the lack of *MDR1* Pgp expression.

The data in Table 1 also indicate that the tumor cells in 7 of 35 brain tumors and various proportions of other (non-brain) tumors were positive for Pgp. In 3 brain tumor cases only, both capillary endothelial and tumor cells were positive for *MDR1* Pgp, indicating that Pgp is expressed independently in these cells. In all 35 brain tumor cases (primary and metastatic), the endothelial cells were Pgp positive at the tumor margin (tumor-brain border) and in the brain further from the tumor.

Figure 1 is an illustration of paraffin sections of a normal brain (Figure 1A) and three cases of glioblastoma (Figure 1, B–D) immunostained for *MDR1* Pgp. Endothelial cells of brain capillaries and the newly formed capillaries in the tumor itself show strong site-specific JSB-1 immunoreactivity. Tumor cells around the capillaries are unreactive. In Figure 1D, the arrow indicates red blood cells without immunostaining, with stained endothelial cells nearby. There was no difference in the relative staining intensity of Pgp in endothelial cells of normal brain *versus* tumor-brain border *versus* the more central region of a glioma. The staining pattern consistently showed that in all positive capillaries every endothelial cell was stained.

Table 2 shows the grade distribution of gliomas and *MDR1* Pgp expression in capillary endothelial cells of the tumors. The majority of cases were highly malignant grade IV gliomas (glioblastomas). In 86% of gliomas (25/29), the endothelial cells of the new capillaries of the tumor exhibited positive JSB-1 immunostaining. All grades lower than glioblastoma multiforme uniformly had positive *MDR1* Pgp immunostaining in endothelial cells of tumor vessels, whereas in glioblastoma multiforme cases the staining pattern was heterogeneous. In 4 cases of grade IV gliomas, endothelial cells of tumor capillaries were unreactive.

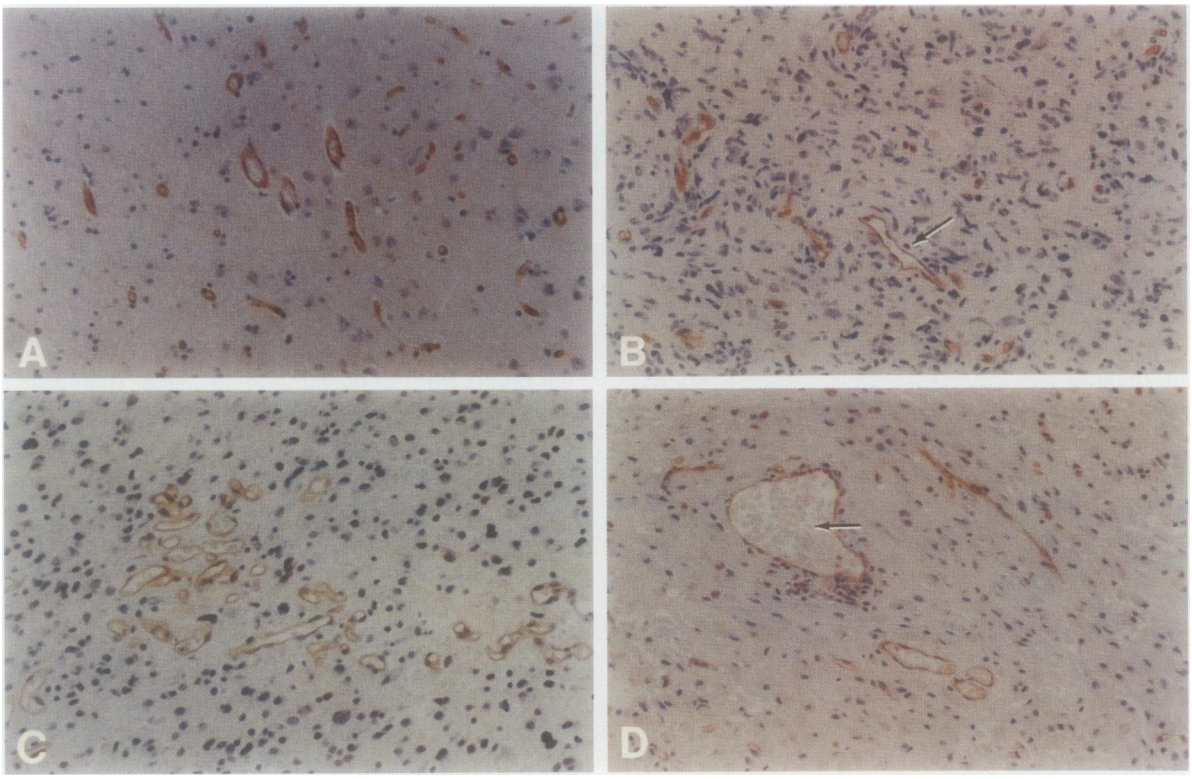

Figure 1. *Immunohistochemical demonstration of* MDR1 *Pgp in capillary endothelial cells by the multilayer sandwich method using JSB-1 in paraffin sections of normal brain* (A) *and three cases of glioblastoma* (B to D) *after formalin fixation. All sections were counterstained with hematoxylin. Original magnification of each panel is the same* (×200). *In* D, *the* arrow *shows red blood cells without immunostaining.*

MDR1 Pgp and anti-factor-VIII showed a very similar immunostaining pattern in vascular endothelial cells in sections of normal brain and in three glioblastoma cases in which almost all capillaries were JSB-1 positive. In another glioblastoma case in which no JSB-1 immunostaining was observed in endothelial cells, there was a positive reaction with anti-factor-VIII demonstrating the presence of vessels without Pgp expression.

Discussion

The induction of vascularization by tumors has been widely accepted for many years but is still not fully understood. This study was focused on only one aspect of this process, namely, the Pgp content of endothelial cells of newly formed vessels in various tumors.

Our results clearly show, for the first time on paraffin sections, that *MDR1* Pgp is expressed not only in normal human brain capillaries at blood-brain barrier sites but also in the large majority of the newly formed capillaries of gliomas and not in other malignant tumors (non-brain). This data confirms already published positive results obtained in frozen sections.[10] Most of the capillary endothelial cells in the gliomas during neoangiogenesis preserve the *MDR1* Pgp expression of normal brain endothelial cells

Table 2. *Grade Distribution of Human Gliomas and* MDR1 *Pgp Expression in Capillary Endothelial Cells of the Tumors*

Gliomas	Positive JSB-1 immunostaining in capillary endothelial cells/cases	Semiquantitative assessment of JSB-1 immunostaining*
Grade I	3/3	s/3+ (2 cases); w/3+ (1 case)
Grade II	3/3	s/3+ (3 cases)
Grade III	1/1	w/3+
Grade IV (glioblastoma)	14/18	s/3+ (9 cases); w/2+ (2 cases); s/1+ (1 case); w/1+ (1 case); s/2+ (1 case)
Grade uncertain	4/4	s/3+ (1 case); s/2+ (1 case); w/3+ (2 cases)
Total	25/29 (86%)	

*Intensity of immunostaining: s, strong as in normal brain capillaries; w, weaker, than normal brain capillaries. Extent of immunostaining: 1+, <5%; 2+, 5 to 75%; 3+, >75% of endothelial cells are stained.

from which they originated. All of the low grade gliomas (lower than glioblastoma multiforme) demonstrated *MDR1* Pgp vascular staining. This observation may indicate that endothelial cells of the neovasculature of low grade gliomas are more likely to express Pgp than those found in high grade glioblastoma multiforme, although the low number of cases do not allow us to make a definitive conclusion. The neovasculature in gliomas is quite variable. Some contain capillaries histologically similar to those seen in normal brain (Figure 1), whereas in others, increased vascularity and endothelial cell proliferation can be seen. We emphasize that *MDR1* Pgp is expressed in the endothelial cells of the capillaries (narrow or dilated) only but not in the larger vessels, which are positive with anti-factor-VIII.

The lack of Pgp immunostaining in endothelial cells in four gliomas (grade IV) and in three metastatic tumors to the brain can be partly attributed to the extensive necrosis or to the absence of capillaries in sections of these highly cellular tumors. A newly described angiogenesis factor, the vascular endothelial growth factor, is a growth factor that binds exclusively to endothelial cells, directly promoting endothelial cell proliferation *in vitro* and *in vivo*.[14,15] Vascular endothelial cell growth factor is produced *in vivo* by glioblastoma cells in response to hypoxia, predominantly in the tumor cells adjacent to areas of necrosis where oxygen supply is low. Vascular endothelial cell growth factor also possesses vascular permeability activity and is thus likely responsible for both the tumor-associated neoangiogenesis and the characteristic leakiness of glioblastoma vessels.[15] The leaking vessels most likely do not contain Pgp in the endothelial cells adjacent to the necrotic areas.

One study reported that some, but not all, commercial lots of JSB-1 (diluted ascites fluid) and C219 contained contaminating anti-A blood group antibodies.[16] We have never observed positive JSB-1 immunoreaction in our recent study, neither in erythrocytes located in capillaries nor in vascular endothelial cells of 169 malignant tumors, findings that would have indicated the problem caused by such contaminating antibody. Furthermore, in our largest patient group (71 breast cancer cases) studied for *MDR1* Pgp, there were 17 patients with blood type A and 7 patients with blood type AB. Despite that, there was no positive JSB-1 immunoreaction in either erythrocytes or endothelial cells located in the stroma of breast cancers, which excludes the possible role of blood group A antibody contamination. In addition, there was no predominance of blood types A and AB patients (4 and 3, respectively) among the 23 Pgp-positive breast cancers. This compelling evidence rules out the possible role of blood group A antibody in the positive JSB-1 immunoreaction.

We have previously documented[11] that capillary endothelial cells of normal brain showed strong positive immunostaining in paraffin sections with JSB-1 (see Figure 2, panel 4, in Ref. 11), but there was no reaction at all with MAbs C494, C219, and MRK16 (see Table 1 in Ref. 11) or with MAbs HYB241 and HYB612 in another study;[17] therefore, we could select JSB-1 only. We fully characterized this assay in our laboratory on paraffin sections of various Pgp-positive and -negative human tissues and model cell lines (see Figure 2 in Ref. 11 for verification). A big advantage of this method, which we applied in our present study, is that no additional frozen tissue is necessary because this immunohistochemical sandwich staining method with MAb JSB-1 allows reliable detection of *MDR1* Pgp in paraffin sections of archived surgical specimens, as it has already been documented in detail in our publication about the new method.[11]

Although the functionality of *MDR1* Pgp in endothelial cells of the neovasculature of brain tumors needs to be examined, drug resistance in malignant brain tumors (primary and metastatic) may result not only from the characteristics of the neoplastic cells but also from Pgp-containing tumor capillary endothelial cells. Pgp in endothelial cells of tumor capillaries and of capillaries located at the brain-tumor border (in residual or recurrent tumors) can exclude chemotherapeutic agents from tumor cells that are located around the capillaries. This idea is strongly supported by a recent ultrastructural study in which Pgp was observed to be specifically expressed on the luminal surface of the endothelium of capillary vessels only in normal brain and glioma by an immunoelectron microscopic analysis.[18] The polarized localization of Pgp suggests that Pgp acts as a one-way efflux pump from the endothelial cytoplasm to the blood in the brain and in the tumor, resulting in a barrier against selected agents. We hypothesize that this phenomenon represents a new mechanism of multidrug resistance in tumors of the central nervous system.

In a recent publication, *MDR1* Pgp was detected in capillary endothelial cells of bladder carcinomas after chemotherapy in three of six post-therapy samples.[19] This preliminary set of data indicates that Pgp can be induced in tumor capillary endothelial cells, which may contribute to drug resistance in non-brain tumors as well.

Many compounds (verapamil, cyclosporin A, cyclosporin D analogue PSC 833, etc) have been

shown to inhibit Pgp activity, and there is currently an intense interest in the potential use of these reversal agents that are under clinical evaluation to reduce the Pgp-mediated drug resistance in human tumors.[20,21] The technique of opening the blood-brain barrier by the administration of Pgp-reversing agents theoretically could improve the Pgp-mediated drug delivery to brain tumors. However, neurotoxicity may be equally elevated. The modulating agent and the cytotoxic drug must be chosen carefully, and the design of the clinical trial should be based on prior preclinical *in vivo* results. This approach is under evaluation in this laboratory and others.[5]

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