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Guest editorial

Does P-glycoprotein play a role in clinical resistance of malignant astrocytoma?

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P-glycoprotein (P-gp) is a 170 kDa transmembrane glycoprotein which plays a significant role in modulating pleomorphic or multiple drug resistance (MDR) in a wide variety of human cancers like renal and colorectal carcinoma. However, its role in modulating drug resistance in other types of cancer is less well defined. The purpose of this review is to critically examine the evidence that P-gp plays an important role in producing drug resistance in astrocytic gliomas. Malignant astrocytoma is clinically resistant to most types of cytotoxic drugs, including those associated with the MDR phenotype and the cross-resistance patterns of short-term cultures derived from malignant glioma are consistent with this phenotype. Consequently, it might be expected that this tumor would express high levels of P-gp. However, immunohistochemical findings from a number of previous studies have provided conflicting data about the expression of P-gp in these tumors, although P-gp has been consistently detected in normal brain in the endothelial cells in cerebral blood vessels and is thought to contribute to the blood–brain barrier phenomena. In order to determine if P-gp contributes to drug resistance in malignant astrocytoma, we undertook a study of P-gp expression in a panel of short-term cultures derived from these tumors in which we determined the *in vitro* chemosensitivity. However, immunocytochemical studies with a panel of antibodies which recognize both internal and external epitopes of the P-gp molecule have consistently failed to show the characteristic membrane staining associated with MDR in any of the cultures, including those markedly cross-resistant to vincristine and doxorubicin. One antibody, JSB-1, showed heterogeneous granular cytoplasmic staining which was unrelated to a particular pattern of drug resistance. This is probably because this antibody cross-reacts with a widely distributed cytoplasmic antigen, pyruvate carboxylase, which is present in abundance in normal astrocytes. The unexpectedly poor specificities of many of the antibodies thought to be specific for P-gp is reviewed in the context of malignant astrocytoma. In conclusion, the role of P-gp in producing drug resistance in malignant astrocytoma is questionable and further studies might more profitably concentrate on the mechanisms of resistance to DNA-damaging agents like the nitrosoureas, methylating agents or platinum-based drugs. [© 1999 Lippincott Williams & Wilkins.]

Key words: Astrocytoma, blood–brain barrier, drug resistance, P-glycoprotein.

Introduction

Malignant astrocytoma in adults presents a formidable clinical challenge. Surgery is almost never curative because of the diffuse invasion of tumor cells into brain parenchyma and radiotherapy, although modestly effective in increasing survival, produces late toxicity with increasing dose. A wide variety of cytotoxic drugs have been used to treat malignant astrocytoma as an adjuvant to radiotherapy with between 20 and 50% of patients achieving a short-term radiological response. However, only the nitrosoureas and procarbazine are effective as single agents in producing increased survival when used as adjuvants to surgery and radiotherapy.[1] It would be of major significance if the mechanism or mechanisms of resistance that contribute to this poor response to chemotherapy could be determined. The mechanisms responsible for producing resistance to alkylating agents have been extensively characterized in malignant astrocytoma, e.g. the role of O^6-alkylguanine-DNA alkyl transferase (O^6-AT) which has been shown to correlate with resistance to chloroethylnitrosoureas like BCNU and CCNU *in vitro*,[2] and with length of survival in astrocytoma patients treated with BCNU-based adjuvant chemotherapy.[3] However, malignant astrocytoma is most often treated with combination chemotherapy usually comprising of a chloroethyl-

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nitrosourea and one or more drugs which commonly include a natural product cytotoxic like vincristine, etoposide or more recently, taxol. These drugs are substrates for P-glycoprotein (P-gp) and it may be that this ATP-dependent transmembrane transporter contributes towards the clinical resistance of patients with malignant astrocytoma to combination chemotherapy. P-gp overexpression has been positively correlated with a poor clinical outcome in other non-CNS cancers,[1-6] and acquired resistance after initial chemotherapy in colon and breast cancer.[7]

In this paper we will review a number of studies aimed at determining the degree of P-gp overexpression in human malignant brain tumors highlighting the problems of interpretation and explain why P-gp expression in malignant astrocytomas is unlikely to be of significant clinically relevance.

Techniques used to detect P-gp in clinical samples

Strategies for detecting P-gp expression have focused on detecting the presence of *MDR1* RNA using Northern blotting or expression of P-gp using immuno-cytochemistry. The former technique relies on the electrophoretic separation of single-strand RNA isolated from cells or tissue are subsequently hybridized with a radiolabeled *MDR1* DNA probe. The size and amount of *MDR1* RNA can easily be determined, but gives only an value averaged across a tumor and normal tissue specimen which does not reflect any heterogeneity in expression between different cell types. It also has the significant disadvantage that large amounts of fresh tissue are required to isolate sufficient RNA, limiting the application of this technique to archival specimens. However, elevated levels of *MDR1* mRNA have been detected in cell lines that were negative using immunohistochemistry.[8] Reverse transcription-PCR (RT-PCR) can be used to amplify up *MDR1* mRNA from total mRNA extracted from biopsy or cell cultures for Northern blotting. The RNase protection assay can be used to show the size and amount of *MDR1* RNA based on the hybridization of a radiolabeled single-stranded *MDR1* DNA probe to total cellular RNA, thus forming DNA-RNA duplexes. The remaining non-hybridized single-stranded RNA is digested away with RNases and the duplexes are analyzed by electrophoresis. This technique can also pick up single base mutations which may alter P-gp function.[9]

Immunocytochemistry has been used to detect and localize P-gp in fixed tissues or cell culture, to interfere with its function and locate potential drug binding sites.[10] There are many antibodies which recognize P-gp,[11] the most commonly used are C219, MRK16 and JSB-1. As these antibodies recognize different epitopes of the P-gp molecule, a panel of two or more antibodies is commonly used in any immunocytochemical study. Immunocytochemistry is at least as specific as the RNase protection assay and more sensitive than Western blotting,[7] but precisely because of this sensitivity the staining patterns are often very difficult to quantify objectively.

Although Western blotting can be used to accurately quantitate levels of P-gp, it suffers from the same disadvantages as Northern blotting with regard to producing only an average P-gp signal over a piece of tissue.

P-gp expression in normal brain

The distribution of P-gp in normal brain appears to be rigidly compartmented (Table 1). It is clear that P-gp does not occur in parenchymal cells in the brain but is restricted to brain endothelial cells and some infiltrating macrophages. P-gp is widely expressed in brain endothelial cells which form the blood-brain barrier (BBB), implying that P-gp plays an important role in removing large molecular weight solutes including cytotoxic drugs from the brain and/or preventing drugs carried in the blood from entering the brain. However, the first of these functions would depend on the reverse orientation of P-gp on the brain side of the endothelial cells, so drugs would be taken up into the endothelial cells then effluxed through normally oriented P-gp into the blood. There is no evidence of this reverse orientation. The second function would require normally oriented P-gp to be expressed preferentially on the luminal side of the endothelial cells only and although this has been demonstrated in rat brain capillaries[16] where 400- to 500-fold overexpression of P-gp was found in luminal membranes, this has not been observed in human brain capillaries. It has been suggested that P-gp may also be expressed on astrocyte foot processes which are directly associated with endothelial cells.[17] However, in enzymatically isolated microvessels free from contaminating astrocytes, P-gp expression is found homogeneously throughout the endothelial cell membranes.[18] This specific spatial expression has only been found in whole capillaries—indeed cultured endothelial cells express P-gp throughout the membrane.[15] It should be noted that P-gp expression is not restricted to brain endothelial cells and that strong P-gp expression has also been demonstrated in endothelium of vessels in other normal tissue including lung, prostate, stomach, intestine and glomeruli.[19]

Table 1. P-gp expression in normal brain

Tissue	Antibodies used	Results	Reference
Fresh-frozen rat brain (four specimens)	MRK16 C219	Four of four samples had capillaries within both the cerebral cortex and cerebellum strongly positive with C219; one of four samples were positive with MRK16	12
Formalin-fixed, paraffin-embedded, normal human brain surrounding astrocytoma (two specimens)	MRK16 C219	One of two samples had macrophages and capillary endothelial cells strongly positive in >75% of cells; one of two samples had endothelial cells which were negative and weak staining of macrophages	13
Fresh-frozen human brain (11 specimens)	C219	Seven of 11 samples were positive in blood vessels, including hippocampus, cerebellum, amygdala and cortex; 11 of 11 samples had negative parenchymal cells	14
Cultured mouse and pig cerebral capillary endothelial cells	C219	All expressed moderate levels of P-gp although staining was heterogeneous	15
Isolated rat brain capillaries	C219	Overexpression of P-gp on luminal membranes only	16

P-gp expression has also been found restricted to two other cell types within the CNS, ependymal cells and the histogenetically related choroid plexus epithelium.[20] Ependymal cells form an epithelial layer lining the ventricular system and although the function of this structure is not clear, it does not seem to be a passive cellular barrier but is thought to regulate the transport of ions, small molecules and water between the cerebrospinal fluid and the neuropil, and may also protect neural tissue from potentially harmful substances,[21,22] physiological roles which are consistent with the expression of P-gp.

Expression of *MDR1* mRNA and P-gp in astrocytic tumors

High levels of *MDR1* mRNA and P-gp are usually found in cancers derived from normal tissues, which themselves have high MDR1 expression, e.g. cancers of the colon, kidney, liver and adrenal gland.[32] However, although normal astrocytic cells do not seem to express appreciable levels of P-gp, astrocytic tumors have been shown to express the *MDR1* gene, although the reported incidence of P-gp expression varies widely between studies,[24,26] with some investigators finding expression in most samples,[27] whilst others find little or no expression.[14] Although the relative differences in sensitivity between Northern blotting and immunocytochemistry[14,25] may account for some of these discrepancies,[24,26] it cannot account for all the differences but suggests that there are major methodological problems with staining using these antibodies. The results from 11 studies investigating the expression of the *MDR1* gene in astrocytoma are shown in Table 2. One of the highest number of P-gp-positive cells (27.6%) in one study was seen in a glioblastoma multiforme (GBM) from a patient who had received previous radiotherapy and chemotherapy, including vincristine,[24] suggesting that this might be an example of acquired resistance to these drugs.

P-gp is expressed in almost all endothelial cells within the tumors,[26] although it was suggested that the presence of an intact BBB presumably where a high degree of cell to cell contact was essential to the expression of P-gp in endothelial cells so where there was more breakdown of the BBB, as in the very malignant GBMs, there may be less P-gp in the endothelial cells. However, in another study five of six GBMs expressed P-gp in endothelial cells,[14] and no difference in P-gp expression was found between endothelial cells of 19 low-grade and 34 high-grade astrocytomas.[29] In a larger study P-gp expression has been demonstrated in the majority of newly formed capillaries of malignant brain tumors, 25 of 29 (86%) grade III and IV astrocytomas, and also in three of six tumors metastatic to the brain, in comparison none of 137 non-brain tumors expressed P-gp in new capillaries.[28]

Expression of *MDR1* mRNA and P-gp in other brain and spinal tumors

The expression of P-gp in oligodendrogliomas and most benign tumors is low, although P-gp was

Table 2. Expression of the *MDR1* gene in astrocytomas

Tissue/source	Techniques	Results	Reference
Two GBM/cell lines GB-1 and U373MG	Northern	26-fold more MDR1 mRNA in GB-1 than U373MG	23
	Southern	Each had one copy of the MDR1 gene	
Six GBM/fresh-frozen specimens	Northern	No expression of MDR1 mRNA	14
	Immuno mAb C219	One of six P-gp-positive in tumor cells; five of six P-gp-positive in endothelial cells	
Five astrocytoma/cell lines 12 GBM, two GrIII Astros/ fresh-frozen specimens	Western mAb C219	One MDR cell line, high P-gp expression; four sensitive cell lines with no P-gp	24
	Immuno mAb MRK16	All tissue samples expressed P-gp with between 6.7 and 27.6% cells positive	
Five GBM, two GrIII Astros/ fresh-frozen specimens	Northern	Three of five GBMs and one of two GrIII Astros expressed MDR1 mRNA	25
	Immuno mAb C219	All expressed P-gp (0.3–15.4% of cells positive)	
22 GBM, 14 GrIII Astros/ fresh-frozen specimens	Immuno mAb HYB-241	Two of 22 GBMs and no GrIII Astros expressed P-gp in tumor cells; 17 of 22 GBMs and 13 of 14 GrIII Astros expressed P-gp in capillaries	26
15 GBM/paraffin embedded	Immuno mAb JSB-1	14 of 15 GBMs expressed some level of P-gp	27
Three pilo Astros, three GrII Astros, one GrIII Astro, 18 GBM/paraffin embedded	Immuno mAb JSB-1	Five of 25 expressed P-gp in tumor cells; 21 of 25 expressed P-gp in endothelial cells; no difference between grades	28
Eight pilo Astros, 11 GrII Astros, nine GrIII Astros, 25 GBM/ paraffin embedded	Immuno mAb JSB-1	P-gp expressed in 0–5% cells of pilo Astros, 0–2% cells of GrII Astros, 0–41% cells of GrIII Astros and 0–54% cells of GBMs	29
Two GrII Astros, nine GrIII astros, 22 GBM/fresh-frozen specimens (total of 104 samples)	RT-PCR	No sample completely negative; heterogeneous expression; strong variation between samples from different areas of same tumor	30
21 GBMs/fresh-frozen specimens and cell cultures derived from same tumor	Immuno mAb C219	Most tumor cells expressed P-gp; no significant difference in expression between sensitive and resistant cultures	31

Gr = grade, Astros = astrocytomas, GBM = glioblastoma multiforme, pilo = pilocytic, Northern = Northern blotting, Southern = Southern blotting, Western = Western blotting, Immuno = Immunocytochemistry.

expressed consistently in a minority of primitive neuroectodermal tumors (PNETs) and virtually all ependymomas (Table 3). Expression of P-gp appears to be particularly common in ependymoma, two series indicate that 29 of 33 (88%) using C219 and JSB-1,[36] and 35 of 42 (83%) using C219 and UIC-2[37] expressed P-gp. Recurrent tumors showed strong expression of P-gp and, in one case where paired samples from the same patient were available, its corresponding primary tumor was negative.[36,37]

Relationship between P-gp expression and drug sensitivity *in vitro*

Doxorubicin (DOX) accumulation has been investigated in three glioma cell lines, one was relatively sensitive to DOX, and two were resistant to DOX and other MDR drugs, although the P-gp status of the cells was not known. No relationship between sensitivity and DOX accumulation was found, but the addition of verapamil which reverses P-gp function significantly increased DOX sensitivity in the resistant cell lines,[38]

Table 3. Expression of the *MDR1* gene in non-astrocytic brain and spinal tumors

Tissue/source	Techniques	Results	Reference
Two oligodendrogliomas/ fresh-frozen specimens	Immunocytochemistry using mAb MRK16	7% of cells expressed P-gp	24
Two oligodendrogliomas, six meningiomas, three schwannomas, one craniopharyngioma/fresh-frozen specimens	Northern blotting (11 specimens)	MDR1 mRNA detected in one of six meningiomas	14
	Immunocytochemistry using mAb C219 (nine specimens)	P-gp expressed in vessels and tumor cells in one of one oligodendroglioma, three of six meningiomas and in vessels only in one of six meningiomas	
29 medulloblastomas/paraffin embedded	Immunocytochemistry using C219, 4E3 and UIC-2	P-gp expressed in 16 of 29 tumors; P-gp expression correlated with adverse clinical outcome	33
16 PNETs/fresh-frozen specimens	Western blotting using JSB-1 MDR1 mRNA by RT-PCR	P-gp expressed in two of 16 specimens; six of 12 specimens expressed MDR1 mRNA; one expressed MDR1 mRNA in recurrent but not in primary	34
18 PNETs, eight ependymomas/fresh-frozen specimens	Immunocytochemistry using mAb MRK16	No P-gp expression in tumor cells; >50% of vessels expressed P-gp in three of eight ependymomas and one of 18 PNETs	35
33 ependymomas/paraffin embedded	Immunocytochemistry using mAb C219 and JSB-1	P-gp expressed in 29 of 33 tumors in some ependymal rosettes and epithelial-lined tubules and clefts and all endothelial cells	36
42 ependymomas/paraffin embedded	Immunocytochemistry using mAb C219 and UIC-2	P-gp expressed in 35 of 42 tumors including all seven recurrent tumors; predominantly cytoplasmic staining	37

PNET=primitive neuroectodermal tumor.

which suggests that sensitization is mediated by two separate mechanisms, one consistent with P-gp acting as an energy-dependent drug pump and one not.

These data clearly have implications for the design of clinical practice regimes for these tumors, suggesting that drugs involved in the MDR phenotype might be of limited value either as single agents or in combination treatment of this tumor.

Response to ACNU, cisplatin and VCR with and without verapamil or nimodipine was compared to the expression of P-gp in 15 short-term GBM cell cultures. Six of the cultures expressed P-gp and could be chemosensitized, seven lines had no P-gp expression and could not be sensitized, and two of 15 did not respond to the calcium channel antagonists even though they expressed P-gp.[27] Interestingly these agents produced sensitization to all the drugs, even though ACNU and cisplatin are not transported by P-gp or implicated in the MDR phenotype, implying that some other resistance mechanism(s) must be present.

Does MDR relate to P-gp expression *in vitro*?

In an attempt to examine the relationship between sensitivity to cytotoxic drugs and expression of P-gp, we have assessed the sensitivity of 21 untreated short-term cultures (passage levels 3–13) derived from adult

high-grade astrocytomas to two drugs involved in the MDR phenotype, VCR and DOX, and one drug, CCNU, used clinically in the treatment of malignant astrocytoma, but whose resistance is not mediated by P-gp. In order to determine if the cultures displayed the classic MDR cross-resistance pattern of resistance to both vinca alkaloids and anthracyclines, the ID_{50} values for VCR and DOX were compared (Figure 1). Instead of a direct relationship between sensitivity to each of the drugs, as might be expected if P-gp expression was the sole mechanism mediating resistance in these cultures, the ID_{50} values clustered into four groups. Group A contains the cultures markedly resistant to both VCR and DOX. Group B contains cultures which retain marked resistance to DOX, but with greater sensitivity to VCR. For the remaining two cultures, the relationship between their sensitivity to VCR and DOX are sufficiently dissimilar as to warrant inclusion in two separate groups. Group C contains a culture markedly sensitive DOX but with intermediate resistance to VCR, as in Group B. Group D contains the only culture markedly sensitive to both drugs. It might be expected that cultures in Groups A and B would express P-gp. All cultures were assessed using fluorescence immunocytochemistry with JSB-1, C219 and MRK16. Different staining patterns were seen with all three antibodies with little agreement between them. Positive staining appeared to suggest the presence of P-gp in the cytoplasm of several of the cultures; however, no cell membrane staining indicative of classical P-gp expression could be detected with any of the antibodies.

All P-gp immunocytochemistry was carried out on subconfluent cultures during exponential growth. There was no apparent difference in the patterns of staining between grade III and grade IV astrocytomas, in agreement with other studies,[25,30] although von Bossanyi and co-workers[29] found the percentage of P-gp-stained cells generally increased with tumor grade.

Patterns of staining with immunocytochemistry

Antibody JSB-1 generally gave strong punctate staining in the cytoplasm, especially in the perinuclear region, although no membrane staining was visible. Most positive cells tended to be the larger cells with the most cytoplasm (Figure 2a and b). Even those cells which were exceptionally sensitive to DOX and VCR showed some cytoplasmic staining with these antibodies, whilst some cultures which were resistant to DOX showed some expression, but if P-gp was present in these cultures they should also be comparatively resistant to VCR and this was not the case. With antibody C219 the staining pattern was different with bright punctate staining less common and the majority of cultures being negative or displaying only faint homogeneous cytoplasmic staining or faint punctate staining, again there was no characteristic membrane localization (Figure 2c). The antibody MRK-16 gave the most bright homogeneous staining between both individual cells and between cultures, with only occasional punctate staining (Figure 2d). These data strongly suggest that

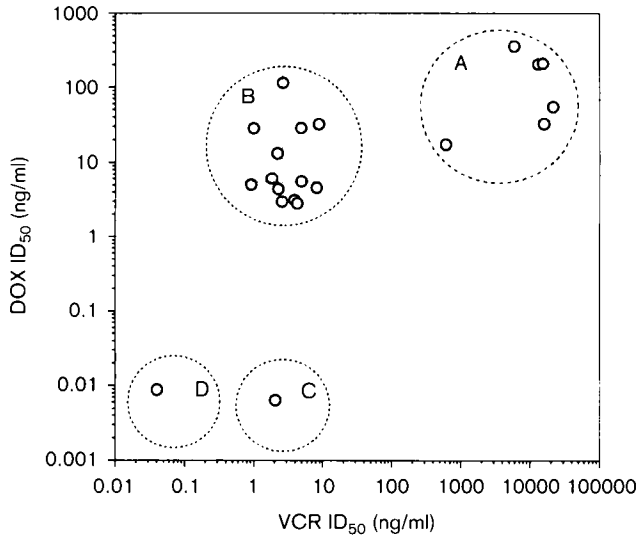

Figure 1. Relationship between VCR and DOX ID_{50} values. Four groups A–D with different resistance patterns are shown. (A) Cultures highly resistant to both DOX and VCR. (B) Cultures highly resistant to DOX and with intermediate resistance to VCR. (C) A culture with intermediate resistance to VCR but sensitive to DOX. (D) A culture sensitive to both DOX and VCR. Crossed lines indicate median ID_{50} values for that group.

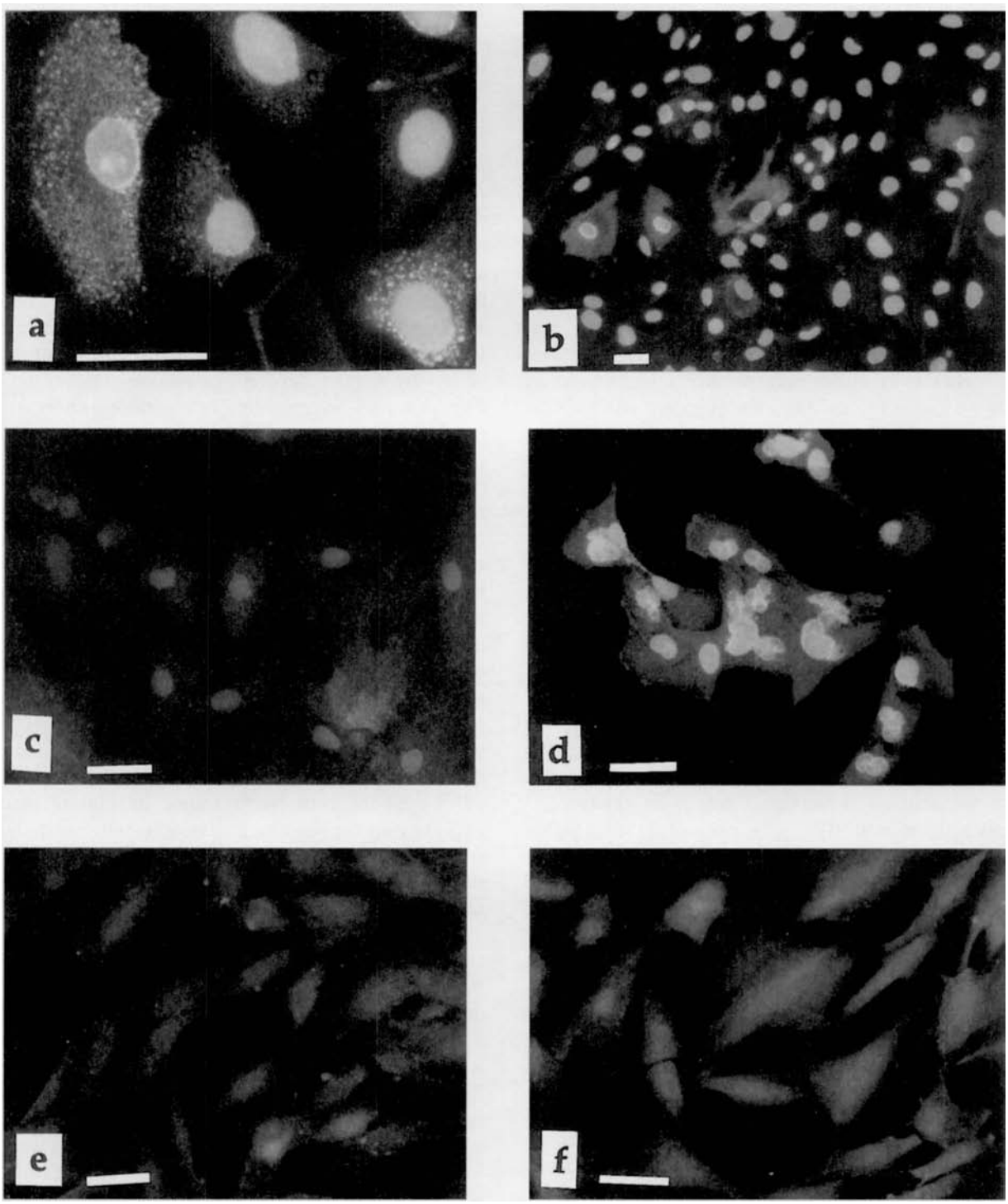

Figure 2. P-gp expression in cultures derived from adult human GBMs. FITC antibody visualization=green; propidium iodide nuclear counterstain=red. (a) Culture IN1902 passage (P)4 with JSB-1, punctate cytoplasm and perinuclear staining. (b) Culture IN1682 P9 with JSB-1, mostly negative with occasional cytoplasmic staining. (c) Culture IN1752 P7 with C219, no positive staining. (d) Culture IN1979 P8 with MRK16, occasional punctate perinuclear staining. (e) Culture IN1760 P8 with JSB-1 general cytoplasmic staining, comparable to (f) culture IN1760 P8 with negative control CT6 (bars=20 μm). Cultures IN1902, IN1682, IN1752 and IN1979 are in resistance pattern Group B, IN1760 is in resistance pattern Group A. All would be expected to express P-gp in the cell membrane.

these antibodies display non-specific binding in cells derived from malignant glioma, as both resistant and sensitive cultures stained positively, and neither group has characteristic membrane staining.

To confirm that P-gp was not being expressed in the cultures, three negative controls were employed: (i) omission of the primary antibody, (ii) inclusion of a teratoma cell line (SuSa) in the panel of cultures which was known not to express P-gp[39] and (iii) an irrelevant mouse IgG1 antibody recognizing guinea pig lymphocytes clone CT6 (Figure 2e and f). Omission of the primary antibody generally gave negative or only faint homogeneous staining; occasionally punctate staining was found. The teratoma cell line stained with all three antibodies with similar staining patterns to the astrocytoma cell cultures—this indicates that the antibodies were either non-specific or recognized an epitope distinct from P-gp. The negative control CT6 clone showed faint homogeneous and/or intermediate punctate cytoplasmic staining.

All three antibodies, however, did produce evidence of cytoplasmic staining. It has been suggested that in cells which exhibit only low levels of resistance, only reverse transcription PCR to amplify *MDR1* RNA is sensitive enough to detect expression.[40] However, in our study some cultures were highly resistant to both DOX and VCR, up to 6×10^4- and 5×10^5-fold more resistant, respectively, than the most sensitive cultures. Uniform positive cytoplasmic staining with JSB-1 and MRK-16 in drug-selected cells that were only 4- to 6-fold resistant to daunorubicin and VCR, and plasma membrane staining in cells which are more than 10-fold resistant to these drugs has been observed,[41] suggesting that if P-gp was present in the highly resistant astrocytoma cultures in our study, it would be detectable by immunocytochemistry. The lack of membrane staining is unlikely to be the result of fixation artefacts as the fixation of the cells and the immunocytochemistry protocols used in the present study were comparable to other studies using a variety of human cancer cell lines. Fixation of cells with acetone,[42] acetone and ethanol,[43] and acetone or methanol,[44] has been shown to be successful in preserving P-gp immunopositivity for all three antibodies, JSB-1, C219 and MRK-16, although formalin or air-drying alone may be preferred for MRK-16.[45]

Problems with the comparison of P-gp expression between studies

P-gp has been found to be expressed in frozen sections or paraffin wax-embedded material of astrocytic tumors and astrocytoma cell lines. However, using C219 and JSB-1, Becker *et al.*[25] found both plasma membrane and cytoplasmic staining in only 0.3 and 15.4% (mean 4.7%), whilst using MRK-16 Matsumoto *et al.*[24] found that only between 6.5 and 21.3% of cells (mean 11.6%) stained positive for P-gp. Others have found that occasional tumors stained almost completely[26] and others have found that virtually all cells in a whole panel of tumors stained positive.[27] Whilst it is possible to dismiss series where there was homogeneous staining of cells across a panel of tumors as a technical artefact, this raises important questions in regard to quantifying immunocytochemical appearance in relation to drug sensitivity. For example, what level of P-gp expression per cell is necessary to confer MDR and what percentage of cells must over-express P-gp for the tumor or culture to be drug resistant? In a review by van der Heyden[7] the minimum definition of P-gp positivity, independent of chemosensitivity data, ranged between studies from a single cell to 30% of cells. Where drug-selected cells are used there does appear to be a correlation between P-gp staining and resistance,[42] but little is known about the relationship in non-selected cells. Levels of cellular drug resistance may correlate to density of P-gp molecules in the membrane rather than the absolute number of P-gp molecules per cell.[46] Using mRNA PCR techniques they showed that parental sensitive KB cells expressed one molecule of *MDR1* mRNA per cell, but 20-fold resistant KB cells selected with COL had 74 molecules per cell. However, the density of actual P-gp molecules was not described and little research has been carried out in this area. Secondly, it is not known what percentage of cells, in a tumor or culture, must express P-gp for resistance to be effected, but if small fractions of cells can render the tumor resistant then this may be related to the ability of the P-gp-expressing cells to repopulate the tumor after drug treatment.

Poor specificity of antibodies used to detect P-gp

It has been found recently that the major obstacle to the detection of P-gp is the unexpectedly poor specificities of most of the antibodies available to detect P-gp. These antibodies, which have been widely used in studies aimed at comparing P-gp expression with drug response or with clinical outcome following chemotherapy, have been shown to have significant cross-reactivities with epitopes unrelated to drug resistance. As early as 1989, concern had been expressed about the use of C219 which had been shown to stain type I (slow twitch) class skeletal muscle fibers, tissue which is known not to contain P-

gp.[12] Additional, apparent cross-reactivity has also been detected in pancreatic acini, seminal vesicle and testis. Using immunoblotting, it was possible to show that C219 reacted with an approximately 200 kDa band in skeletal and cardiac muscle which migrated in the same position as heavy chain myosin. More recently, this situation has been further complicated by a report which demonstrates that C219 also cross-reacts with the 185 kDa c-*erbB2* product in MDA-MB-435 human breast cancer cells transfected with the c-*erbB2* cDNA.[47] Peptide sequence analysis has shown that C219 recognizes a shared epitope between these two molecules.[47] However, the erbB2 receptor system is of much less importance to astrocytoma biology than to other cancers.[48]

JSB-1, which shows marked cytoplasmic staining in our panel of cell cultures, also appears to have a major cross-reactivity to a protein not involved in drug resistance. The antibody appears to cross-react with a 130 kDa protein which is present in rat liver mitochondrial inner membrane/matrix fractions. This appears to be pyruvate carboxylase, an enzyme present in large amounts in mitochondria.[49] Another P-gp 'specific' antibody, C494, also appears to cross-react with pyruvate carboxylase, although JSB-1 and C494 appear to recognize distinct but closely proximate epitopes in this molecule.[50] It is therefore of significance that brain endothelial cells which often stain with JSB-1 in tissue section have 3- to 5-fold more mitochondria than systemic endothelial cells, necessary to support their numerous energy-dependent transport mechanisms.[51]

The use of these antibodies for staining cultured cells, frozen sections or archival material has greatly been compromised. In situations where strong staining is confined to the cell membrane this is likely to reflect specific staining for P-gp. However, the significance of weak homogeneous cytoplasmic or granular cytoplasmic staining is more difficult to interpret. It may well be that this reflects staining of pyruvate carboxylase. This highly conserved enzyme is found in a wide variety of prokaryotes and virtually all eukaryotic tissues,[52,53] including astrocytes.[54] It plays an important role in gluconeogenesis by catalyzing the formation of oxaloacetate from pyruvate and HCO_3^-. It is interesting that despite the high levels which have been found in astrocytes, that staining with JSB-1 in the glioma cell cultures proved to be extremely heterogeneous. Some cultures had very faint, homogeneous, cytoplasmic staining whilst other cultures had very strong punctate staining either in the cytoplasm or in the perinuclear region. There was often marked heterogeneity in the degree of staining within a single culture. It may be that this is a reflection that the phenotypic and genotypic heterogeneity of malignant glioma, and that this extends to expression of pyruvate carboxylase, or it may be that some, low level expression of P-gp is present in these cultures. If this does occur, it is quite plain that it is restricted to the cytoplasm of glioma cells, perhaps in the Golgi apparatus or as discrete cytoplasmic vesicles as described below.

Although these antibodies cross-react with proteins of different molecular weight to P-gp, care must be taken in Western blotting studies because pyruvate carboxylase with a molecular weight of 130 kDa could be confused with the 130 kDa core-glycosylated P-gp, the precursor of P-gp which is converted into its mature form by *N*-linked glycosylation.[55]

Cell biological factors which complicate the accurate detection of P-gp in astrocytoma *in vitro*

There may be additional biological factors which could account for the differences between P-gp expression *in situ* and *in vitro*. Perhaps cells which express P-gp differ in some biological characteristics which make their survival *in vitro* more difficult. For example, the disruption of the three-dimensional structure brought about by processing cells for culture might in some way down-regulate the expression of P-gp. This may be the result of changes in cell-cell or cell-extracellular matrix contact. Certainly, changes in the expression of integrins have been reported in MCF-7 human breast cancer cells made resistant to DOX,[56] although the effect of this on P-gp expression was not determined. In another study, Kaaijk *et al.*[57] produced so-called organotypic multicellular spheroids from fragments of fresh glioma tissue obtained during surgery on seven patients. Although these cells had never grown as monolayers, about 5% of cells within the spheroids expressed P-gp. It may be because *in vitro* culture conditions remove some of the intercellular signals like growth factors which *in situ* might maintain P-gp expression. For example, insulin-like growth factor I up-regulates P-gp expression leading to the inhibition of apoptosis in MCLM colon cancer cells,[58] and retinoic acid and C6 conditioned medium have been shown to increase the levels of P-gp in an immortalized rat brain endothelial cell line.[59] It is also possible, although there is as yet no direct experimental evidence, that P-gp-expressing cells, although present in the tumor, do not adapt to culture readily and are progressively lost during the initial stages of cell culture. This may be because they grow more slowly *in vitro*, have lower plating efficiencies or that

media supplemented with fetal calf serum and consequently rich in platelet-derived growth factors are not optimal in maintaining the long-term viability of cells which express P-gp.

Although we have found no apparent membrane expression of P-gp, cytoplasmic staining was common, initial studies suggested that JSB-1 produced a cytoplasmic staining pattern which was thought to be associated with early development of low level MDR,[11,12] although these cells with cytoplasmic staining were later found to have no *MDR1* mRNA by PCR which suggests that the antibody was recognizing an antigen other than P-gp.[60] However, more recently, additional evidence has been produced to suggest that the site of action of P-gp may not solely be at the cell surface. Molinari *et al.*[61] have shown that P-gp can be found in the Golgi apparatus of MDR cells but not sensitive cells. In the resistant cells, DOX accumulates cytoplasmically in the Golgi apparatus and this accumulation can be reversed by ATP-depletion or treatment with verapamil, indicating that it is P-gp which sequesters the drug. This can occur in cells which do not express P-gp on their cell surfaces.[62] In human myeloma cells selected in the presence of DOX and verapamil, DOX appears to accumulate in the cytoplasm in contrast to the parental, sensitive cells where it accumulates almost exclusively in the nucleus. This process correlates with a 2.5-fold increase in P-gp in the cytoplasm.[63] The intracytoplasmic structures which are responsible for this are probably P-gp-containing cytoplasmic vesicles, distinct from endocytic vesicles, in which the P-gp molecules are orientated in such a way that drugs are transported and accumulate within the interior of the vesicles.[64] The net result of this sequestration of cytotoxic drug within the cytoplasm is a reduction of the net amount of free drug within the cytoplasm which could potentially reach the nucleus, effectively producing drug resistance. It has been suggested recently that the acidic pH of organelles contributes to the sequestration of drugs like DOX and that P-gp because of its function as a chloride channel is responsible for maintenance of acid pH within these vesicles.[65] It is interesting that in our study, MRK 16, an antibody directed against an external epitope of P-gp, seems to produce some cytoplasmic staining in permeabilized cells. As yet, there have been no reports of confounding cross-reactivities with this antibody.

Conclusions

It is almost impossible to find agreement between studies assessing the expression of P-gp in astrocytomas, be it in formalin fixed, fresh frozen or cell culture. There are major problems with the specificities of the available antibodies, and little agreement between studies on the best way to qualify and quantify results. The difficulties with techniques for RNA and DNA extraction are now being recognized with the careful microdissection of tumor and normal tissue, and removal of all vasculature essential. There is also little evidence that any P-gp present is functional, i.e. elicits MDR. However, all studies agree that P-gp is over-expressed in the endothelial cells of such tumors and therefore surely interferes with drug delivery. This must be a major reason why natural product drugs are not routinely used to treat high-grade astrocytomas. Attempts to competitively block P-gp at these sites might be of clinical utility in improving drug penetration into brain tumors, although it may have unpredictable sequela in normal brain. The role of P-gp as a major mechanism of natural product resistance in malignant astrocytoma in adults is questionable and studies of drug resistance in these tumors might more productively concentrate on resistance mechanisms to effective drugs like the chloroethylnitrosoureas, methylating agents like temozolomide or platinum-based drugs.

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