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Ultrastructural localization of P-glycoprotein on capillary endothelial cells in human gliomas

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Abstract The P-glycoprotein (P-Gp) encoded by the human multidrug-resistance gene *MDR1* has been suggested to play certain roles in the blood-brain barrier (BBB). However, the detailed mechanism of the activity of P-Gp in multidrug-resistance (MDR) remains unclear in human glioma. We examined the localization of P-Gp in human glioma by immunohistochemical (IHC) and immunoelectron microscopic (IEM) methods with anti P-Gp monoclonal antibodies (C219, MRK16). We also examined *MDR1* expression in primary glioma and xenografts by reverse transcription-polymerase chain reaction (RT-PCR) with human *MDR1*-specific primers. The IHC study showed no P-Gp expression on tumour cells but it was present on capillary endothelial cells and IEM analysis showed definitive localization on their luminal surface. *MDR1* gene expression was detected in eight primary glioma and three normal brain specimens by RT-PCR, but not in glioma xenografts. The lack of *MDR1* expression in these cells appears to be a consequence of the replacement of the original human stroma, including blood vessels, by murine stroma in glioma xenografts. The unique distribution of P-Gp on the capillary blood vessels was confirmed in human glioma by the results of immunohistochemical and molecular biological studies.

Key words Multi-drug resistance · P-glycoprotein
Human glioma · Endothelial cells · Blood-brain barrier

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Introduction

Gliomas generally show cross-resistance to multiple anticancer agents, rendering chemotherapy ineffective [9, 20]. Several mechanisms including the blood-brain barrier (BBB) have been proposed to account for multidrug-resistance (MDR) in glioma [26]. The essential structures of the BBB have been suggested to comprise ultrastructural features such as tight junctions [21, 28].

Recently, a membrane-associated P-glycoprotein (P-Gp) has been suggested as one of the components of the BBB [33]. Some studies have shown that over-expression of the P-Gp encoded by a human multidrug-resistance gene, *MDR1*, induces the MDR phenotype in human neoplasms in vitro [5, 6, 13, 29, 35]. P-Gp functions as an energy-dependent efflux pump for the transport of lipophilic anticancer agents. In some solid tumours such as those of the colon and kidney, P-Gp has been reported to be overexpressed in tumour cells [10, 12]. However, the precise mechanism of P-Gp-mediated MDR in glioma is unclear, although many studies have reported the over-expression of P-Gp/*MDR1* in glioma [4, 7, 16, 25, 34]. The major issues are whether and how the over-production of P-Gp is related to MDR in glioma.

We investigated the morphological distribution of P-Gp in glioma by immunohistochemical (IHC) and immunoelectron microscopic (IEM) analyses. We also examined *MDR1* expression in glioma by reverse transcription-polymerase chain reaction (RT-PCR) assay with human *MDR1*-specific primers. In the present study, we discuss the localization of P-Gp and the mechanisms of the intrinsic MDR phenotype in human glioma.

Table 1 Profiles of clinical specimens and xenografts of human glioma (*FA*, Fibrillary astrocytoma; *AA*, anaplastic astrocytoma; *GM*, glioblastoma multiforme; –, only surgery; *Ra*, radiation therapy; *Ch*, chemotherapy; *NE*, not examined; *A*, alive; *D*, died; *m*, month)

Clinical specimens					
Case	Age	Sex	Histology	Treatment	Outcome
1	78	F	FA	–	D (4 m)
2	51	M	GM	+ (Rd)	D
3	29	M	FA	+ (Ra)	NE
4	64	M	AA	–	D (1 m)
5	64	F	FA	+ (Ch, Rd)	A (21 m)
6	18	M	FA	–	A (19 m)
7	69	M	GM	+ (Ch, Rd)	A (18 m)
8	55	M	GM	–	D (8 m)
Xenograft	Age	Sex	Histology	Prior chemotherapy	
GL-3-JCK	47	M	GM	–	
GL-5-JCK	50	M	GM	–	
GL-22-JCK	55	M	GM	–	

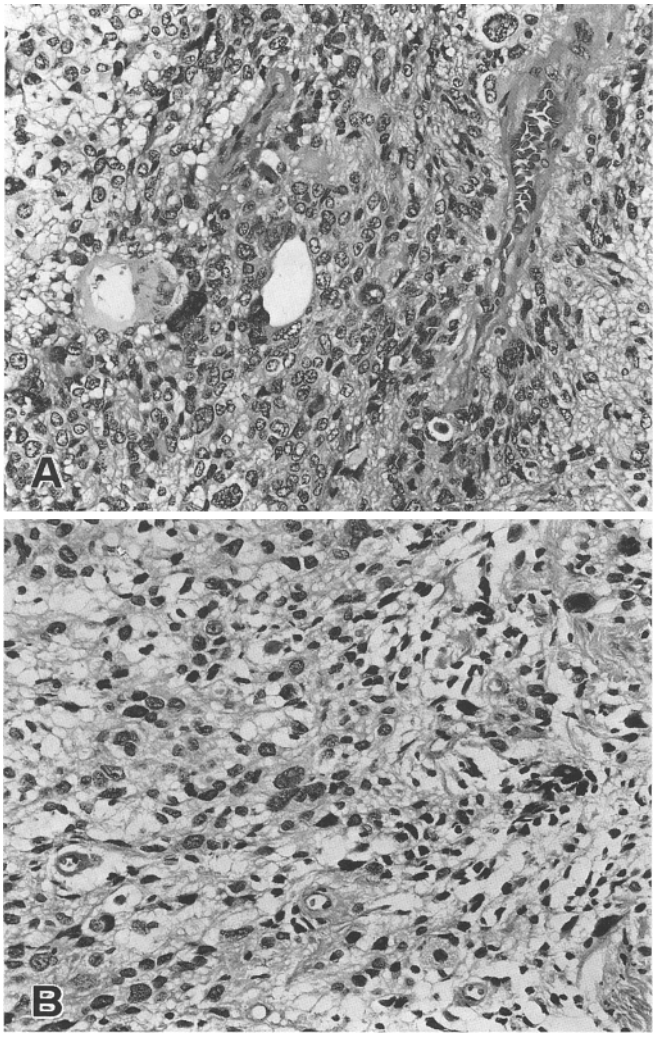

Fig. 1 Histopathology of the primary glioma and the xenografts. Sections shown were stained with haematoxylin and eosin (×300). **A** Glioblastoma multiforme (case 8). The glioma was xenotransplanted as GL-22-JCK. **B** Glioma xenograft (GL-22-JCK). No significant morphological differences were apparent between primary tumour and xenografts

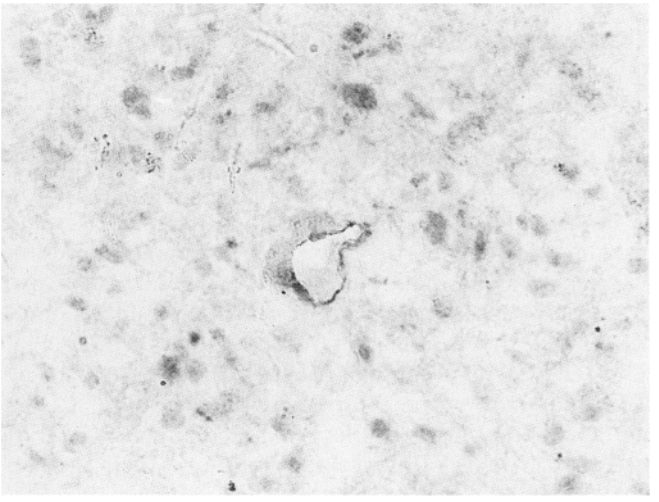

Fig. 2 Immunohistochemical staining (C219) of glioma and normal brain tissue (×600). Positive staining was observed in the tumour vessels in astrocytoma (Case 3)

Materials and methods

Eight primary brain tumour (glioma) specimens were obtained at surgery from patients with untreated glioma, after obtaining informed consent. These specimens were fixed with 4% paraformaldehyde, embedded and frozen in OCT compound (Tissue-Tek, Miles Inc. Elkhart, Ind., USA) for both IHC and IEM analyses (Table 1). Fresh tumour specimens were also rapidly frozen at – 80° C until used for further molecular biological analysis [30]. A surgical specimen (case 8) was used for the establishment of a GL-22-JCK xenograft. Normal brain specimens were obtained at autopsy from three patients without apparent cerebral lesions.

Three human glioma xenografts were established from primary glioma specimens obtained from patients in whom there had been no previous chemotherapy. The xenografts were maintained by serial subcutaneous transplantation in nude mice (BALB/c-nu/nu; Clea, Japan Inc., Tokyo). We obtained xenografts from mice under deep anaesthesia. No significant morphological differences were observed between xenografts and primary tumours (Fig. 1A, B).

Fresh frozen sections (4 μm-thick) for IHC were processed by indirect immunostaining methods, employing anti-P-Gp monoclonal antibodies C219 (Centcore, Inc., Malvern, Pa., USA) or MRK16. Sections were incubated with anti-P-Gp antibodies and horseradish peroxidase (HRP)-conjugated sheep anti-mouse im-

Table 2 Expression of P-glycoprotein (P-Gp) and *MDR1* gene

Sample	P-Gp[a]	MDR1 gene[b]
Normal brain	3/3	3/3
Primary glioma	8/8	8/8
Glioma xenograft	0/3	0/3

[a] The expression of P-glycoprotein (P-Gp) was detected only on endothelial cells by both immunohistochemical and immunoelectron analyses

[b] The expression of the human *MDR1* gene was detected by reverse transcription-polymerase chain reaction assay with human *MDR1*-specific amprimers

munoglobulin (Amersham, UK). Reaction products were visualized by incubation in 0.02% 3,3'-diaminobenzidine (DAB) containing 0.005% hydrogen peroxide [15, 18, 31]. We used the MDR cell line KB8–5 as a positive control and its parent chemotherapy-sensitive cell line KB3-1 as a negative control for immunostaining.

Thick sections (8 μm) for IEM were incubated with anti-P-Gp antibody (C219 and MRK16) overnight at 4° C, after inhibition of endogenous peroxidase activity and non-specific binding. After visualizing the reaction with DAB, the sections were post-fixed with 2% osmium tetroxide and embedded in Quetol 812 resin for preparation of ultrathin sections. Ultrathin sections were examined using an electron microscope at 80 keV (JEOL, 100C) [32].

MDR1 gene expression was determined by RT-PCR by a modification of the procedure described previously [3, 24]. RT-PCR was designed to yield a 243 bp fragment specific for the *MDR1*-cDNA by using primers from either side of an intron to prevent amplification of contaminating genomic DNA. We used amprimers for the human β2-microglobulin (β2m) gene to assess RNA quality by evaluating housekeeping gene expression. PCR products, separated by gel electrophoresis (3% agarose, NuSieve GTG, FMC Bioproducts, Rockland, Me., USA) and blotted onto membranes (Zeta Probe, BioRad), were detected by hybridization with synthetic oligonucleotide probes labelled with ^{32}P [19].

Results

We investigated the localization of P-Gp in human glioma and normal brain tissue by IHC. Staining patterns in gliomas and in normal brain tissue were similar with both C219 and MRK16 anti-P-Gp monoclonal antibodies. P-Gp immunoreactivity was consistently localized to the walls of capillary blood vessels in both human brain tissue and in glioma (Fig. 2), whereas neither normal

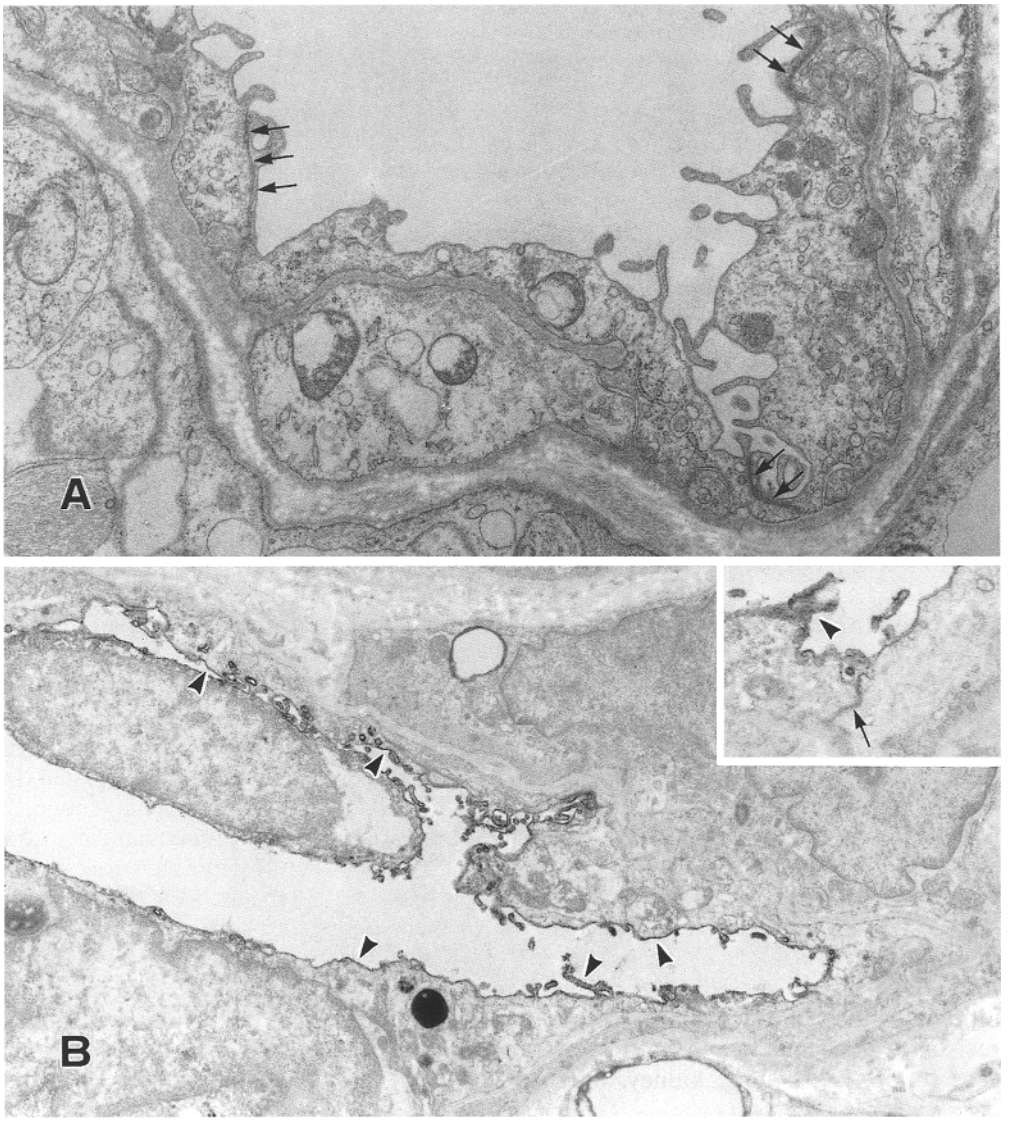

Fig. 3 Electron microscopic analyses of glioma (Case 3, astrocytoma). **A** Electron micrograph showing well-developed tight junction (*arrow*) and basement membrane (×17750). **B** Immunoelectron microscopic analysis (anti-P-Gp Ab, C219) of astrocytoma showed the localization of P-Gp immunoreaction products (*arrowhead*) on the luminal surface of capillary endothelial cells (×8880). **Inset**: ×12 430 (*arrowhead*, microvilli, *arrow*, tight junction)

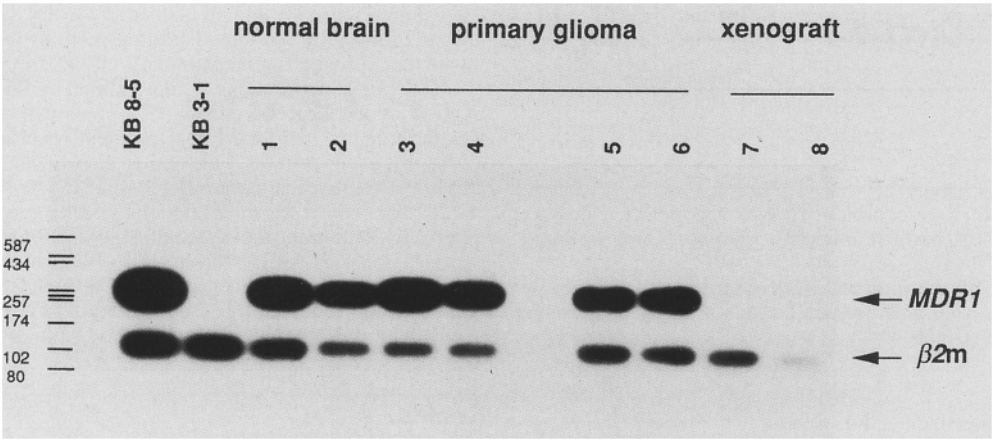

Fig. 4 *MDR1* gene expression determined by reverse transcription-polymerase chain reaction. *MDR1* (243 bp) and human β2m (120 bp) were amplified by PCR with reverse transcribed cDNA from 500 ng of the total cellular RNA. DNA size markers (pUC19 DNA digested with Hae III) are also shown. All the human glioma and normal brain specimens examined expressed *MDR1*, while no expression was detected in human glioma xenografts. Glioma xenograft GL-22-JCK was established from an *MDR1*-expressing primary glioma (case 8). KB8-5, MDR epidermal carcinoma cell line; KB3-1, drug sensitive epidermoid carcinoma cell line. Lanes 1 and 2, normal brain tissue; lanes 3–6, primary glioma (lane 3, case 1; lane 4, case 3; lane 5, case 5; lane 6, case 8), lanes 7 and 8, glioma xenograft (lane 7, GL-22-JCK; lane 8, GL-3-JCK)

neural cells (neurons, glial cells) nor glioma cells were positive for P-Gp. In glioma xenografts, P-Gp immunoreactivity was observed in neither tumour cells nor stromal vessels (Table 2).

Electron microscopic observation showed well-developed tight junctions and microvilli in the endothelial cells of capillary blood vessels in glioma specimens (Fig. 3A) similar to those observed in normal brain tissue. The general ultrastructural features of glioma were well-preserved. Immunoreaction products for P-Gp were seen on the surface folding structures in endothelial cells, and elevated expression of P-Gp was also observed on the membranes of pinocytic vesicles in these cells. P-Gp was observed to be specifically expressed on the luminal surface of the endothelium of capillary vessels in glioma by IEM (Fig. 3B, Table 2). No significant differences in P-Gp-staining patterns on capillary endothelial cells were observed between normal brain tissue and glioma tissue.

MDR1 expression was observed in eight human glioma and in three normal brain tissue specimens, whereas no human glioma xenografts (three xenografts) expressed *MDR1* (Fig. 4, Table 2). No significant differences in *MDR1* expression levels were observed between these glioma specimens. The glioma in case 8 showed apparent *MDR1* gene expression in primary tumour specimens, while its xenograft (GL-22-JCK) had lost *MDR1* expression (Fig. 4). The human *MDR1*-specific amprimers did not amplify the murine multidrug-resistance gene mdr1a cDNA from normal murine tissues (brain, heart, lung, kidney, liver, spleen; data not shown). Thus, we avoided the amplification of mdr1a gene transcripts that may have contaminated the tumour xenografts [2, 8, 14, 17].

Discussion

Human glioma usually shows MDR to various lipophilic anticancer agents such as vincristine (VCR), doxorubicin (DOX) [9, 20]. The existence of the BBB between neural tissue and systemic blood circulation may contribute to MDR in intra-cranial tumours. However, the detailed mechanisms of MDR are not clear in glioma. Some putative ultrastructural features including tight junctions have been considered to constitute the BBB [21, 28], and recently, membrane-associated P-glycoprotein (P-Gp) has been suggested to be one of the components of the BBB [33]. However, the essential structures or roles of the BBB are not clear.

In the present study, we observed that P-Gp production was limited to endothelial cells from both human glioma and normal brain specimens, whereas no production of P-Gp was noted in human glioma xenografts by IHC with anti-P-Gp monoclonal antibodies. We also confirmed that P-Gp was preferentially expressed on the luminal side of the endothelial cells in glioma by IEM. RT-PCR assay with human *MDR1*-specific primers demonstrated common expression of *MDR1* gene in glioma specimens, while glioma xenografts lacked *MDR1* expression. We also demonstrated that *MDR1* expression disappeared in glioma xenografts (GL-22-JCK) established from an *MDR1*-expressing primary glioma (case 8). The results suggested that the loss of P-Gp expression in human glioma xenografts was due to the replacement of the original human stroma, including intra-cranial vessels, by murine stroma with subcutaneous blood vessels.

It has been reported that P-Gp overexpression in tumour cells themselves is correlated with clinical prognosis in such solid tumours as colon and kidney cancers [11, 36, 37]. In addition, we also confirmed previously that colon and pancreatic cancer xenografts retained *MDR1* expression [1]. Some studies have suggested that overexpression of P-Gp in glioma cells also contributes

to the MDR phenotype [4, 23, 25], although others have reported that P-Gp is expressed only on the endothelial cells of glioma stromal vessels [7, 16]. We demonstrated restricted P-Gp expression on endothelial cells in glioma by both IHC and IEM analyses, and confirmed this pattern of expression with molecular biological evidence using unique glioma xenografts. Gliomas are composed histopathologically of heterogeneous elements including endothelial cells. In certain gliomas, the endothelial cells show remarkable proliferation, although it is unclear whether the endothelial proliferation is neoplastic or reactive in such cases. Previous studies have shown P-Gp expression in glioma cells using glioma cell lines in vitro. However, in these studies the precise glial origin of the cell lines were not confirmed at the molecular biological level [4, 23, 25]. The possibility of the vascular origin of the cell line should be excluded in such studies. The discrepancies, therefore, in results regarding of P-Gp expression between the present and these previous studies may be due to contamination by glioma cells and neoplastic endothelial cells in specimens. We are currently investigating P-Gp expression in gliomas with vascular sarcomatous components.

Our previous studies have shown that glioma xenografts, transplanted subcutaneously into nude mice, were uniformly sensitive to such anticancer drugs as VCR and DOX in vivo [3, 22]. However, these agents are clinically ineffective against human glioma [20]. The unique localization of P-Gp on endothelial cells of stromal vessels may explain the discrepancy between the clinical and the in vivo experimental drug sensitivities of gliomas. Its restricted localization suggests that P-Gp acts as a one-way efflux pump from the endothelial cytoplasm to the blood in the brain, resulting in a barrier against selected agents. Our results suggest that the intrinsic MDR phenotype observed in human glioma is due to P-Gp expression on the nutritional blood vessels.

We did not examine the expression of P-Gp or *MDR1* in primary glioma or glioma xenograft tissues after chemotherapy, and so it is not clear whether the over-expression of P-Gp is inducible in these tumour cells. Further analysis of P-Gp expression after chemotherapeutic treatment will provide more information concerning the mechanism of MDR in glioma.

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