Jpn. J. Cancer Res. **92**, 211–219, February 2001

Involvement of the Multidrug Resistance Protein 3 in Drug Sensitivity and Its Expression in Human Glioma

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The multidrug resistance protein (MRP) family belongs to the ATP-binding cassette superfamily (ABC) of transporters, which are involved in ATP-dependent transport of hydrophobic compounds. One of the MRP family, *MRP1*, is partially associated with the multidrug resistance phenotype in brain tumors. In this study, we asked whether another MRP family gene, *MRP3*, could affect drug sensitivity to anticancer agents in human glioma cell lines and clinical glioma specimens. We first produced two antisense transfectants by introduction of antisense *MRP3* cDNA into the glioma cell line NHG2, which endogenously expresses *MRP3*. The two *MRP3* antisense transfectants showed 2- to 5-fold increases in drug sensitivity to etoposide and cisplatin compared with NHG2 cells, but their sensitivity to vincristine or nitrosourea was not changed. Two *MRP3* cDNA sense transfectants of pig kidney cell lines showed 4- to 6-fold drug resistance to etoposide, but only 1.4- to 1.5-fold to cisplatin. We next compared the mRNA levels of four ABC transporters, multidrug resistance 1 (*MDR1*), *MRP1*, *MRP2* and *MRP3* in clinical samples, including 34 patients with gliomas, by quantitative RT-PCR analysis. In some of the clinical samples, increased expression of *MRP1* and *MRP3* was apparent in malignant gliomas. *In situ* hybridization revealed that glioma cells were stained with *MRP3* probe. MRP3 may modulate drug sensitivity to certain anticancer agents in human gliomas.

Key words: Glioblastoma — MRP subfamily — ABC transporter — Anticancer agents

Two representative genes for ATP-binding cassette (ABC) transporter superfamily proteins, multidrug resistance 1 (*MDR1*) and multidrug resistance protein 1 (*MRP1*), mediate acquisition of a multidrug resistance phenotype through altered membrane transport of various anticancer agents in tumor cells.[1, 2] *MDR1* encodes P-glycoprotein (Pgp), which specifically catalyzes ATP-dependent efflux of the vinca alkaloids (vincristine and vinblastine), anthracyclines (doxorubicin and daunomycin), colchicine, taxols, and epipodophyllotoxins (etoposide and teniposide).[3] On the other hand, the MRP1 protein is involved in ATP-dependent efflux of epipodophyllotoxins, anthracyclines, vinca alkaloids and heavy metals.[4]

In addition to *MRP1* genes, other *MRP* family genes, *MRP2*, *MRP3*, *MRP4*, *MRP5* and *MRP6*, are known to be expressed in various human cell types and tissues.[5] *MRP2*/*cMOAT* is expressed in human liver and duodenum.[6, 7] Mutations of *MRP2* have been identified in patients with the Dubin-Johnson syndrome.[8, 9] *MRP3* gene expression has been found in liver, duodenum, colon, adrenal gland, and pancreas,[10, 11] while *MRP4* is expressed at low levels in lung, kidney, urinary bladder, and gall bladder.[5] *MRP5* is expressed in various tissues with relatively high expression in skeletal muscle and brain.[5] Concerning the possible involvement of *MRP* family genes in drug resistance to anticancer agents, *MRP2* and other related genes appear to influence drug sensitivity to vinblastine, doxorubicin, cisplatin, and camptothecin derivatives.[12–14] Forced expression of *MRP3* resulted in acquisition of resistance to etoposide and methotrexate.[15, 16] Both *MRP4* and *MRP5* transcripts have been identified in cancer cells,[17] but whether their products are associated with drug sensitivity is not yet clear.

Glioblastoma multiform is a highly malignant primary neoplasm of the human central nervous system, that is refractory to antineoplastic chemotherapy. Various agents including nitrosourea, etoposide, cisplatin, vincristine, and doxorubicin have been used to treat human gliomas, but their therapeutic efficacy has been unsatisfactory.[18, 19] Resistance to chemotherapy is partially associated with expression of the *MDR1* gene in the blood-brain barrier[20, 21] and in the glioma cells.[22] However, *MDR1* is only expressed in 10 to 20% of human gliomas. *MRP1* is also expressed in some human glioma cell lines and clinical samples.[23]

In this study, we tested whether *MRP3* was coupled to resistance to certain anticancer agents by transfection of *MRP3* sense and antisense cDNA. We next investi-

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gated expression of *MRP* family genes in human surgical specimens.

MATERIALS AND METHODS

Clinical samples Surgical specimens of human hippocampus and gliomas were obtained from patients with epilepsy and glioma at Kyushu University Hospital. Histologic diagnosis and tumor grades were determined according to the WHO classification after staining of specimens with hematoxylin and eosin. Specimens included 18 glioblastomas (grade IV), 8 anaplastic astrocytomas (grade III), and 8 low-grade astrocytomas (grade II). Total RNA from the human brain cortex was obtained from OriGene Technology Inc. All human samples were obtained under an Institutional Review Board-approved protocol, with subjects having provided informed consent.

Drugs and chemicals Cisplatin was donated by Bristol-Myers Squibb KK (Kanagawa). Vincristine, 3-[4-amino-2-methyl-5-pyrimidinylmethyl]-1-(2-chloroethyl)nitrosourea (ACNU) and doxorubicin were purchased from Sigma Chemical Co. (St. Louis, MO). Etoposide was obtained from Nippon Kayaku Co. (Tokyo). Lipofectamine was purchased from Life Technologies Inc. (Bethesda, MD). The labeled drug [^{3}H]vincristine sulfate was obtained from Amersham International Ltd. (Buckinghamshire, UK) and [^{3}H]etoposide was obtained from Moravek Biochemical (Brea, CA).

Cell lines and cultures Six human glioma cell lines, derived from human glioma, and LLC-PK1 (polarized pig kidney epithelial cell line) were used in this study. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 60 μg/ml kanamycin as described previously.[24)]

RNA extraction and northern blot analysis Northern blot analysis was performed as described previously.[24)] To determine the expression of the individual *MRP1*, *MRP2*, *MRP3*, we established specific probes for each *MRP* family member by polymerase chain reaction (PCR) amplification. Levels of mRNA were quantified densitometrically with a Fujix BAS 2000 bio imaging analyzer (Fuji Photo Film Co., Tokyo).

Quantitative reverse transcriptase-PCR (RT-PCR) For quantitative RT-PCR, we used real time 'TaqmanTM' technology and a Model 7700 Sequence Detector (Applied Biosystems, Foster City, CA) as described.[25)] Four primer pairs and four TaqMan probes for *MDR1*, *MRP1*, *MRP2* and *MRP3* were designed using the primer design software "Primer Express" (Perkin-Elmer Applied Biosystems). To avoid amplifying contaminated genomic DNA, primer pairs were placed in a different exon and the probe was placed at the junction between two exons. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; TaqMan GAPDH control reagent kit) were purchased from Perkin-Elmer Applied Biosystems. Sequences for the TaqMan probes and primers were described previously.[26)]

***In situ* hybridization** For *in situ* hybridization, DIG (digoxigenin)-labeled sense and antisense RNA probes were synthesized with T7 RNA polymerase from 621-bp template cDNA using DIG-RNA labeling Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Prior to hybridization, 6-μm thick sections were treated with proteinase K (1 μg/ml) at 37°C for 5 min and postfixed with 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) for 20 min. Thereafter, the sections were treated with 0.2 *N* HCl to inactivate internal alkaline phosphate and acetylated with 0.25% acetic anhydride in 0.1 *M* triethanolamine (pH 8.0) for 10 min. The pretreated sections were dehydrated, air-dried, and hybridized overnight with DIG-labeled RNA probe in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 600 m*M* NaCl, 10 m*M* dithiothreiol (DTT), 0.25% sodium dodecyl sulfate (SDS), and 250 μg/ml *Escherichia coli* tRNA) at 50°C. After hybridization, each section was washed with 5× SSC (1× SSC=0.15 *M* NaCl-0.015 *M* sodium citrate) briefly and then with 50% formamide-2× SSC for 30 min at 50°C. The sections were then washed with 2× SSC for 10 min, followed by 0.2× SSC for 20 min twice at 50°C. Detection of hybridization was performed immunohistochemically with alkaline phosphate-conjugated Fab fragment of anti-DIG antibody using DIG-Nucleic Acid Detection Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Finally, the sections were counterstained with methylgreen.

Western blot analysis Immunoblotting was performed as described previously.[14)] In brief, aliquots of whole-cell lysates (50 μg) were electrophoresed on 6% SDS-polyacrylamide gels and transferred onto Immobilon-P membranes. Antibodies against *MRP1* (QCRL-1),[27)] *MRP3*[15)] and DNA topoisomerase II α (TopoII α)[28)] were used as primary antibodies. Membranes were incubated with antibodies for 1 h at room temperature and then with horseradish peroxidase-linked second antibody for 1 h at room temperature. Membranes were developed by chemiluminescence following the ECL protocol (Amersham International).

Construction of sense MRP3 and antisense *MRP3* or antisense *MRP1* expression vector A CMV-SPORT-*MRP3* clone,[11)] was digested with *Sal*I to isolate a 638-bp fragment containing 621 bp of coding sequence and 17 bp of 5′ noncoding fragment sequence of human *MRP3* gene. The fragment was subcloned into the *Sal*I sites of the pCI-neo vector. pJ3Ω-*MRP1* clone was digested with *Acc*I and *Not*I to isolated 720 bp 5′ coding sequence of human *MRP1* gene. This fragment was also subcloned into the *Sal*I site of the pCI-neo vector. A CMV-SPORT-*MRP3* clone[11)] was digested with *Not*I to isolate the full construct

of human *MRP3* gene. This fragment was subcloned into the *Not*I sites of the pIRES-neo vector to obtain a sense *MRP3* expression vector.

Transfection with sense *MRP3*, antisense *MRP3* or *MRP1* expression vector Exponentially growing NHG2 cells or LLC-PK1 cells (5×10^6) were washed with phosphate-buffered saline (PBS) and placed in serum-free medium. Cells were incubated in the presence of 50 μg of Lipofectamine, and 10 μg of sense *MRP3* or antisense *MRP1* or *MRP3* expression vector for 12 h, and washed with fresh medium. Cells were then incubated in selection medium containing 800 μg/ml G418 for 3 to 4 weeks. Two stable antisense transfectants, NHG2/MRP3AS5 and NHG2/MRP3AS7, that showed reduced cellular *MRP3* levels, one stable antisense transfectant, NHG2/MRP1AS1 and two stable sense transfectants PK1/MRP3S4 and PK1/MRP3S6 were selected from the G418-resistant transfectants. We also isolated G418-resistant mock transfectants, NHG2/Vec and LLC-PK1/Vec, produced by transfection of the vector alone.

Chemosensitivity testing by colony formation assay Cell survival was determined by plating 3×10^3 cells from the glioma cell lines in 35-mm dishes in the absence of drugs. Various drugs were added 24 h later. After incubation for 7 days at 37°C, colonies were counted after Giemsa staining. All drugs were freshly prepared in physiologic saline or dimethyl sulfoxide. All control experiments were done by adding equivalent volumes of saline or dimethyl sulfoxide. The 50% lethal dose (IC_{50}) for each cell line was determined from the dose-response curves.

Cellular accumulation of vincristine, etoposide and cisplatin Accumulation of vincristine and etoposide was determined with [^{3}H]vincristine and [^{3}H]etoposide as described previously.[14] Cells (2×10^5) were plated in 24-well plates and incubated for 48 h at 37°C. The plates were chilled for 15 min, washed twice with ice-cold PBS and incubated at 37°C for 60 min in 200 μl of buffer (serum-free DMEM and 20 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.5) containing 20 n*M* [^{3}H]vincristine (9.60 Ci/mmol and 0.25 μCi/μl) or 1 μ*M* [^{3}H]etoposide (900 mCi/mmol and 1 μCi/μl). Cells then were washed three times with ice-cold PBS and resuspended in 400 μl of 0.25 *M* NaOH at 37°C for 30 min. The resulting cell lysates were mixed thoroughly with 4 ml of Scintisol EX-H (Wako Chemicals, Osaka), and the radioactivity was determined in a liquid scintillation counters. All results were normalized to cell numbers.

The intracellular concentration of cisplatin was measured as described previously.[14] Exponentially growing cells were incubated for 2 h with 100 μ*M* cisplatin, washed twice with ice-cold PBS, harvested, and sonicated. The platinum concentration was determined by flameless atomic absorption spectrophotometer (Atomic Absorption Spectrophotometer 180-70; Hitachi, Tokyo). All results were normalized to cell protein content.

RESULTS

Expression of *MRP1* and *MRP3* in human gliona cells at various levels We first examined expression of *MRP1*, *MRP2* and *MRP3* genes as well as one Pgp family gene, *MDR1*, in six human glioma cell lines by northern blot analysis. We determined that probes for *MRP2* and *MRP3* could specifically detect the respective mRNA species in stable *MRP2* and *MRP3* cDNA-containing transfectants of LLC-PK1 cells.[29, 30] No expression of the *MDR1* gene was apparent in any of the cell lines (data not shown). Consistent with our previous study,[24] *MRP1* was expressed in all cell lines except IN157, and *MRP2* was not expressed in any of the six cell lines (Fig. 1). *MRP3* was expressed in all cell lines. Of three *MRP* family genes, *MRP1* and *MRP3* genes, but not *MRP2*, were expressed in one or more of the human glioma cell lines. The six human glioma cell lines employed in this study showed varying levels of sensitivity to a number of anticancer agents.[24]

Establishment of antisense and sense *MRP3* cDNA transfectants To determine if the *MRP3* gene is responsible for the resistance of these glioma cells to certain anticancer drugs, we established cell lines producing reduced levels of MRP3 protein by introducing an *MRP3* antisense expression plasmid into NHG2 cells. We first established two stable antisense transfectants, NHG2/MRP3AS5 and NHG2/MRP3AS7, which produced 40% and 45% of the level of MRP3 protein produced by NHG2 cells, respectively (Fig. 2A). In contrast, the two transfectants showed similar levels of MRP1 protein to those in both NHG2 and NHG2/Vec (Fig. 2A). We also established the stable

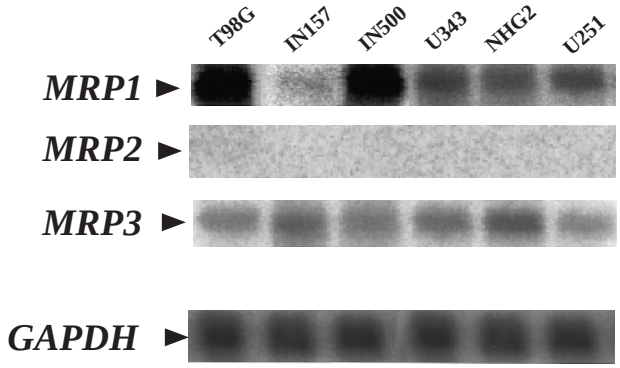

Fig. 1. Comparison of cellular mRNA levels for the three *MRP* family genes in six human glioma cell lines. Northern blot analysis was performed with each specific *MRP* family gene probe. Arrows indicate transcripts of *MRP* family genes. *GAPDH* probe was used as a control.

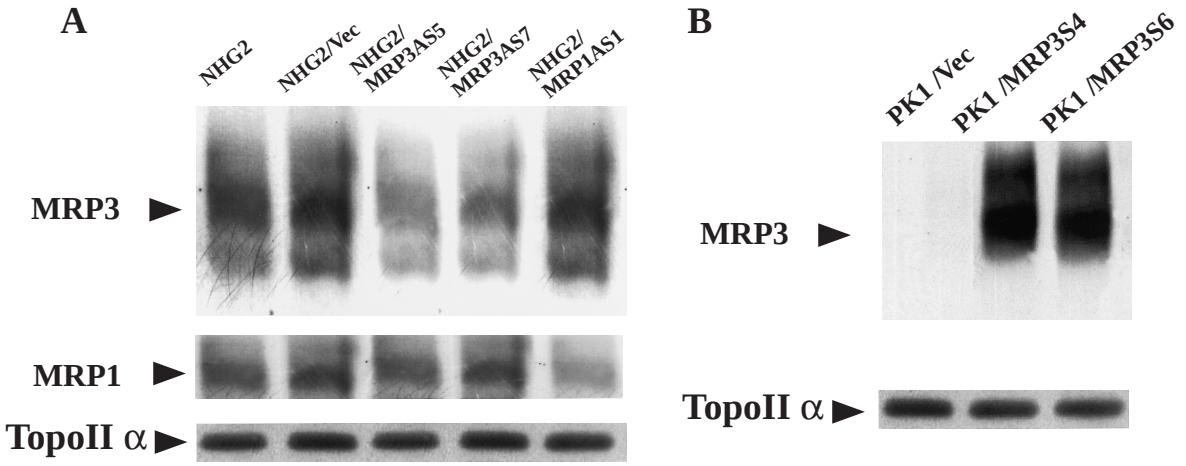

Fig. 2. A. Comparison of cellular protein levels of MRP3 in NHG2, NHG2/Vec, two *MRP3* cDNA antisense transfectants, NHG2/MRP3AS5 and NHG2/MRP3AS7, and *MRP1* cDNA antisense transfectant NHG2/MRP1AS1. Western blot analysis for MRP3, MRP1 and TopoII α was performed with specific antibodies as described in "Materials and Methods." B. Comparison of cellular protein levels of MRP3 in PK1/Vec and sense transfectants PK1/MRP3S4 and PK1/MRP3S6. Western blot analysis for MRP3, and TopoII α was performed with specific antibodies as described in "Materials and Methods."

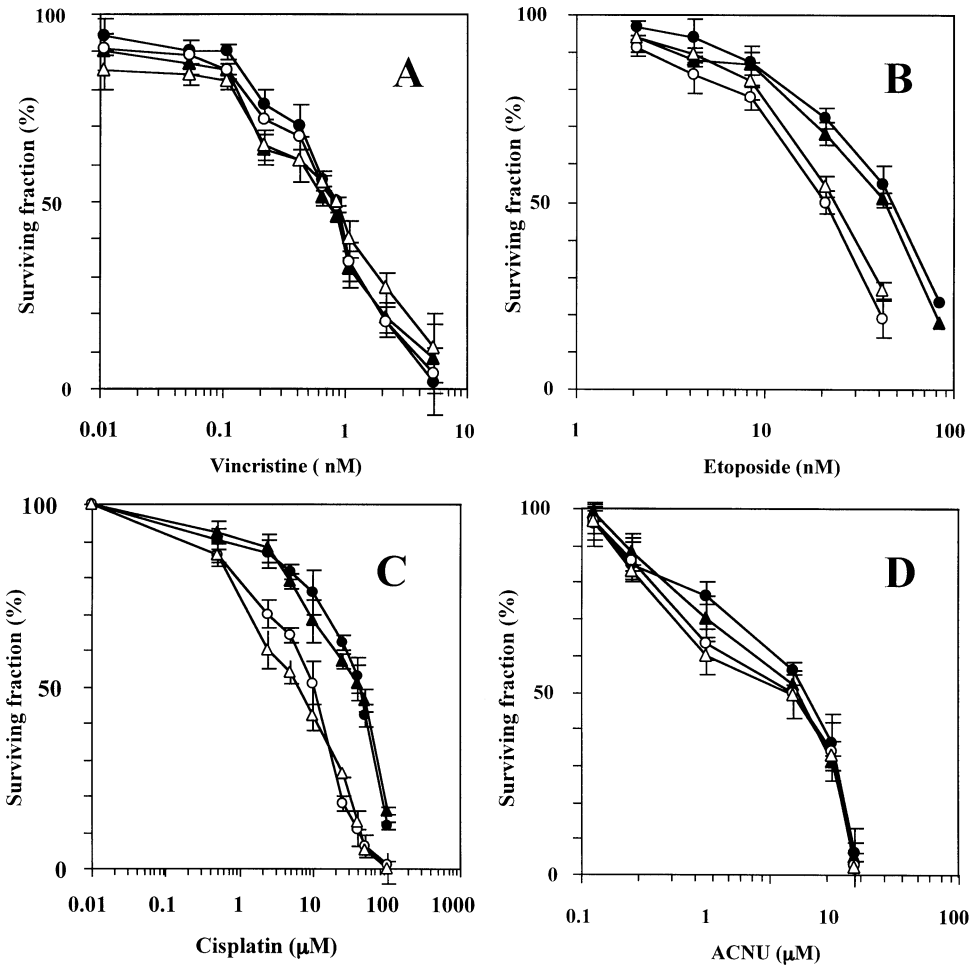

Fig. 3. Comparison of dose-response curves to vincristine (A), etoposide (B), cisplatin (C), and ACNU (D) in *MRP3* antisense transfectants and empty vector transfectants in NHG2 cells. NHG2 (●), NHG2/Vec (▲), NHG2/MRP3AS5 (○), NHG2/MRP3AS7 (△). One hundred percent corresponds to the number of colonies in the absence of any drug. Each point is the average of three separate experiments; bars, SD.

Table I. Comparison of Drug Sensitivity to Anticancer Drugs in *MRP3* (NHG2/MRP3AS5 and NHG2/MRP3AS7) and *MRP1* (NHG2/MRP1AS1) Antisense Transfectants and Their Counterparts

Drug	NHG2	NHG2/Vec	NHG2/MRP3AS5	NHG2/MRP3AS7	NHG2/MRP1AS1
Etoposide	46.1±2.3[a)]	42.5±9.2	21.2±4.6*	24.3±5.3*	34.6±2.4*
	(1.0)	(0.92)	(0.46)	(0.53)	(0.75)
Vincristine	0.86±0.14	0.85±0.27	0.90±0.3	0.88±0.21	0.17±0.07*
	(1.0)	(0.98)	(1.04)	(1.02)	(0.2)
Cisplatin	41.5±7.7	43.0±2.7	12.3±0.8*	6.8±0.5*	40.67±5.1
	(1.0)	(1.03)	(0.30)	(0.16)	(0.98)
ACNU	6.79±0.13	6.81±0.21	6.56±0.35	6.95±0.55	6.65±0.78
	(1.0)	(1.0)	(0.96)	(1.02)	(0.97)

a) The (IC_{50}) values for drugs are the mean±SD from six separate experiments. The IC_{50} concentrations for etoposide and vincristine (n*M*), and cisplatin and ACNU (μM) are presented. In parenthesis, relative resistance was calculated by dividing the mean IC_{50} for each cell line by that shown by NHG2 cells .
* Significantly different ($P<0.05$) from NHG2/Vec (unpaired Student's *t* test).

Table II. Drug Accumulation in *MRP3* Antisense Transfectants and Their Parental Cells

Drug	NHG2	NHG2/Vec	NHG2/MRP3AS5	NHG2/MRP3AS7
Etoposide	0.12±0.01[a)]	0.12±0.05	0.39±0.12*	0.51±0.26*
	(1.0)	(0.92)	(5.8)	(4.3)
Vincristine	0.62±0.15	0.65±0.09	0.68±0.21	0.63±0.12
	(1.0)	(1.04)	(1.04)	(1.02)
Cisplatin	2.5±0.3	2.3±0.5	6.0±0.7*	5.9±0.4*
	(1.0)	(0.92)	(2.4)	(2.36)

a) Cellular accumulations of the three drugs are presented as pmol/10^6cells (etoposide and vincristine) and pmol/mg protein (cisplatin). In parenthesis, relative accumulation was calculated by dividing the intracellular amounts of vincristine, etoposide and cisplatin by the amounts in NHG2 cells. Each value represents the mean of results from three separate experiments.
* Significantly different ($P<0.05$) from NHG2/Vec (unpaired Student's *t* test).

MRP1 antisense transfectant NHG2/MRP1AS1, which produced 40% of the level of MRP1 protein produced by NHG2 cells and a similar level of MRP3 protein to NHG2 cells (Fig. 2A).

We next established cell lines which overexpressed MRP3 protein by introducing *MRP3* sense expression plasmid into LLC-PK1 cells. Since MRP3 was expressed in all six glioma cell lines (see Fig. 1), we used LLC-PK1 cells as recipient for the MRP3 sense transfection experiment. Western blot analysis showed that two stable sense transfectants, PK1/MRP3S4 and PK1/MRP3S6, overexpressed MRP3 protein of 190–200 kD, but PK1/Vec did not.

Drug sensitivity to anticancer agents and drug accumulation in *MRP3* cDNA antisense and sense transfectants

We next determined the sensitivity to etoposide, vincristine, cisplatin, and nitrosourea (ACNU) of NHG2/MRP3AS5, NHG2/MRP3AS7, NHG2 and NHG2/Vec cells by means of colony formation assays (Fig. 3). The *MRP3* antisense transfectants were 2- and 5-times more sensitive to the cytotoxic effects of etoposide and cisplatin, respectively, than their parental counterparts. In contrast, all four cell lines showed similar sensitivities to vincristine and ACNU. Furthermore, the *MRP1* antisense transfectant, NHG2/MRP1AS1, was 1.3- and 5-times more sensitive to etoposide and vincristine, respectively, than the parental counterparts, but the sensitivity to cisplatin and ACNU was not changed (Table I).

We also examined whether MRP3 changes cellular accumulation of vincristine, etoposide and cisplatin. Cellular accumulation of cisplatin was about 2-fold higher in NHG2/MRP3AS5 and NHG2/MRP3AS7 cells relative to their parental counterparts, NHG2 and NHG2/Vec (Table II). Accumulation of etoposide in NHG2/MRP3AS5 and NHG2/MRP3AS7 increased about 4 times over that in NHG2 or NHG2/Vec. However, accumulation of vincristine was at similar levels in mock transfectants and parental cells.

Two MRP3 sense transfectants, PK1/MRP3S4 and PK1/MRP3S6, were 3.4- and 4.4-times more resistant to etoposide than PK1/Vec, but showed almost similar sensitivity to vincristine (Table III). PK1/MRP3S4 and PK1/MRP3S6 showed only a slight increase of 1.4- to 1.5-times in resistance to cisplatin. Cellular accumulation of etopo-

Table III. Comparison of Drug Sensitivity to Anticancer Drugs in MRP3 Sense Transfectants and Their Counterparts

Drug	PK1/Vec	PK1/MRP3S4	PK1/MRP3S6
Etoposide	0.527±0.13[a)] (1.0)	1.78±0.46* (3.4)	2.34±0.22* (4.4)
Vincristine	10.8±1.4 (1.0)	9.72±2.2 (0.9)	12.9±6.6 (1.2)
Cisplatin	86±4.7 (1.0)	126±17.7 (1.5)	150±12.3 (1.4)

a) The (IC_{50}) values for drugs are the mean±SD from six separate experiments. The IC_{50} concentrations for etoposide and vincristine (μM), and cisplatin (n*M*) are presented. In parenthesis, relative resistance was calculated by dividing the mean IC_{50} for each cell line by that shown by PK1/Vec.
* Significantly different ($P<0.05$) from PK1/Vec (unpaired Student's *t* test).

side in PK1/MRP3S4 and PK1/MRP3S6 was decreased to about 30% of that in PK1/Vec cells, and that of cisplatin in both sense transfectants was only slightly reduced by about 5 to 10% from the control counterpart (data not shown).

Expression of *MRP* family genes in clinical samples We examined the mRNA levels of *MDR1*, *MRP1*, *MRP2* and *MRP3* in surgically removed gliomas from 34 patients and normal brain tissue (2 hippocampus and 1 cortex), using quantitative RT-PCR with specific primers and probes. The data were standardized against *GAPDH* mRNA levels. Fig. 4 showed the expression of four ABC transporters *MDR1*, *MRP1*, *MRP2* and *MRP3* in both gliomas and normal brain tissue. Relatively high expression of *MRP1* was observed in normal brain tissues but very low expression of *MDR1*, *MRP2* and *MRP3* (group A in Fig. 4). These was no apparent expression of any of the transporter genes in grade II tumors (group B in Fig. 4). However, expression of *MRP1* and *MRP3* was significantly increased in some malignant gliomas (grade III and IV) (group C in Fig. 4). Expression of *MRP1* and *MRP3* was upregulated in 13 and 10 of the grade III and IV samples, respectively, in comparison with normal brain tissues or grade II tumors (Fig. 4). No increased expression of *MDR1* and *MRP2* mRNA levels was observed in malignant gliomas.

To determine whether *MRP3* mRNA was specifically expressed in glioma cells, two glioma (grade IV) samples

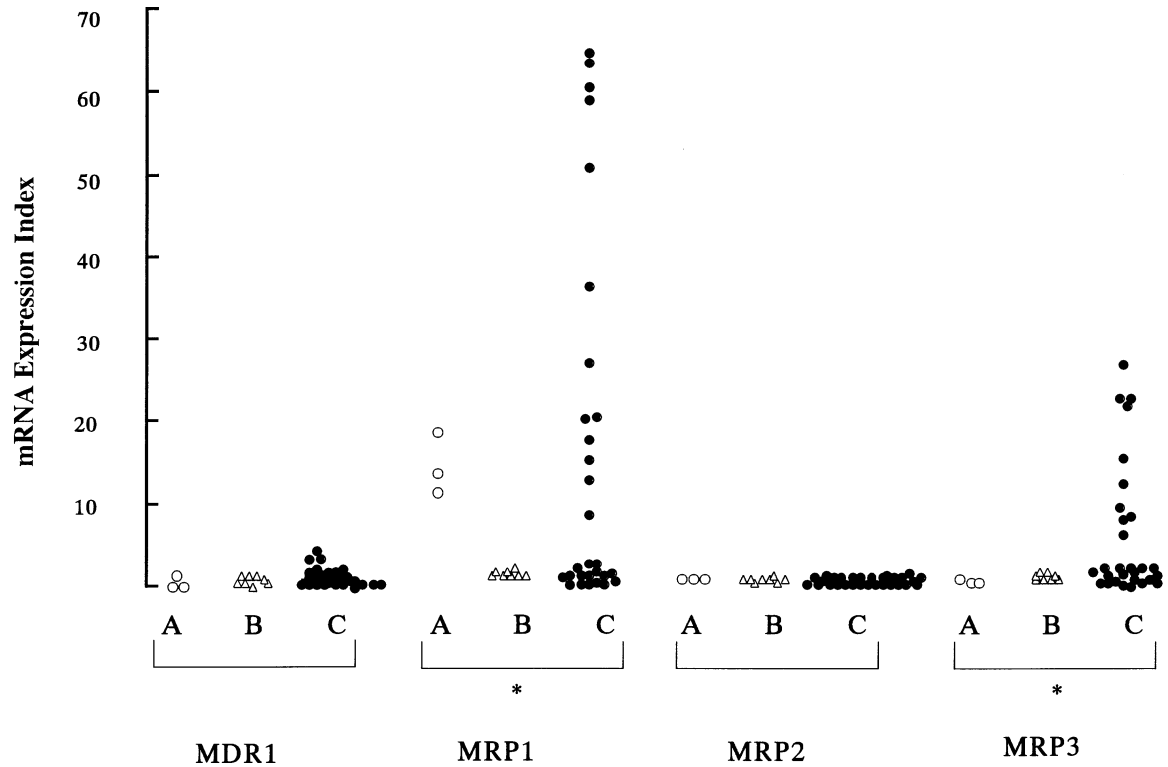

Fig. 4. Comparison of mRNA levels of four ABC transporter genes in normal brain tissue (A), grade II glioma (B) and malignant gliomas (grades III and IV) (C). To determine the mRNA levels of the four ABC transporters, we used mRNA expression index, which is mRNA expression level standardized to that of GAPDH. Normal brain tissue ($n=3$) (○), grade II glioma ($n=8$) (△), malignant glioma (grades III and IV) ($n=26$) (●). Significance of differences was evaluated using the Kruskal-Wallis test: $*P<0.05$.

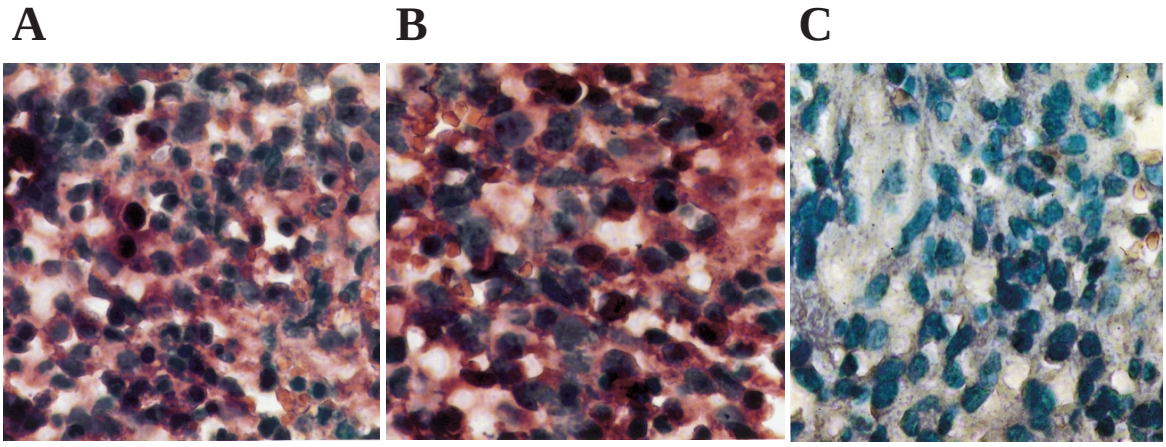

Fig. 5. *In situ* hybridization of grade IV gliomas using DIG-labeled antisense *MRP3* probe. Glioma cells mainly show *MRP3* mRNA with a cytoplasmic staining pattern in case 1 (A) and case 2 (B). When the sections were treated with RNase in the process of washing, the result was the same, though the entire signal was reduced. Negative controls included hybridization with the sense RNA probe and absence of anti-DIG antibody. None of the controls showed a positive signal in case 1 (C). Finally, the sections were counterstained with methylgreen. Magnification, ×400.

were examined by *in situ* hybridization analysis. Glioma cells were strongly stained with antisense probe in the sections of two glioblastomas (grade IV), whereas other cells, including endothelial cells, were not or were only weakly stained (Fig. 5, A and B). When the sections were treated with RNase during washing, the result was the same, though the entire signal was reduced. Virtually no positive signal was observed from any cell in the sections hybridized with the sense probe (Fig. 5C).

DISCUSSION

Of three *MRP* family genes, *MRP1* and *MRP3* were expressed in human glioma cell lines and clinical glioma specimens (Figs. 1 and 4). *MRP1* is known to be expressed in normal brain tissues.[5] However, unlike *MRP1*, expression of the *MRP2* and *MRP3* has not been detected in normal brain tissue.[7, 11] *MRP1* is also expressed in some human glioma cell lines and clinical samples.[23] Consistent with those studies, we observed no apparent expression of *MRP2* and *MRP3* in normal brain tissue (group A in Fig. 4). *MRP3*, which has 56% homology of amino acid sequence with *MRP1*, was expressed in some glioma specimens (group B in Fig. 4). *In situ* hybridization revealed that glioma cells were positively stained with *MRP3* mRNA among the clinical samples which highly expressed *MRP3* gene (Fig. 5). Expression of *MRP3* thus appears to be induced ectopically in malignant cells in the human brain.

Our previous study[14] demonstrated that antisense *MRP2*/*cMOAT* cDNA transfectants could increase sensitivity to cisplatin as well as vincristine, doxorubicin and camptothecins. Cui *et al.*[31] found that forced expression of human *MRP2* cDNA in human embryonic kidney cells and Madin-Darby canine kidney cells confers resistance to vincristine, doxorubicin, cisplatin and etoposide. Studies in our laboratory have also determined that forced expression of human *MRP2* cDNA in Chinese hamster ovary cells and LLC-PK1 cells results in acquisition of resistance to vincristine, camptothecin derivatives, doxorubicin and cisplatin, but not to etoposide.[29, 30] Resistance to cisplatin appeared to be partly induced by forced expression of *MRP2* in various nonpolarized and polarized cell lines. Moreover our recent study has demonstrated that expression of *MRP2* is increased in colorectal cancers compared with non-cancerous tissues and also that drug sensitivity to cisplatin is closely associated with *MRP2* mRNA levels in colorectal cancers.[26] MRP2 thus might play a critical role in limiting sensitivity to cisplatin *in vitro* as well as *in vivo*.

We next examined if drug sensitivity to cisplatin, etoposide, ACNU and vincristine was altered by overexpresion of MRP3, as assayed by colony formation after continuous exposure to drugs. Young *et al.*[32] recently reported that *MRP3* contributes to acquisition of a phenotype resistant to etoposide and cisplatin in human lung cancer cell lines. In agreement with this, introduction of antisense *MRP3* cDNA altered drug sensitivity to both etoposide and cisplatin (Table I). Forced expression of sense *MRP3* cDNA into LLC-PK1 cells has been found to alter drug sensitivity to etoposide, with little if any change in sensitivity to cisplatin (Table III). Kool *et al.* reported that forced

expression of *MRP3* cDNA could significantly alter sensitivity to epipodophyllotoxins (etoposide and teniposide) and methotrexate.[15] Our present study, together with previous studies, suggests that MRP3 plays a key role in both sensitivity to etoposide and efflux activity. It remains unclear if MRP3 itself is directly involved in resistance to cisplatin. Intracellular glutathione might play an essential role in MRP3-meditated drug sensitivity to cisplatin, as noted previously. Unlike MRP1 and MRP2, MRP3 protein appears to show poor affinity to glutathione and its conjugates. Further study is necessary to determine whether *MRP2* or *MRP3* is directly involved in sensitivity to cisplatin, in addition to etoposide, with or without glutathione or glucuronide.

In conclusion, in 34 clinical samples of human gliomas, expression of either *MRP1* or *MRP3* was increased in 10 to 13 malignant samples of grades III and IV in comparison with normal brain tissues or grade II tumors. However, no apparent expression of *MRP2* or *MDR1* was observed in gliomas, including malignant samples. Expression of *MRP3* as well as *MRP1* might modulate drug responses to some chemotherapeutic agents in gliomas.

ACKNOWLEDGMENTS

We thank Akihiko Nishie, Morimasa Wada and Takanori Nakamura in our laboratory for fruitful discussions. This study was supported by a Grant-in-Aid for Scientific Research on Priority Area of ABC Proteins by the Ministry of Education, Science, Sports and Culture, Japan.

(Received July 25, 2000/Revised November 7, 2000/Accepted November 22, 2000)

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