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Expression of Multidrug Resistance Protein-3 (Multispecific Organic Anion Transporter-D) in Human Embryonic Kidney 293 Cells Confers Resistance to Anticancer Agents[1]

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ABSTRACT

Multidrug resistance-associated protein (MRP)1 and canalicular multispecific organic anion transporter (cMOAT)/MRP2 are ATP-binding cassette (ABC) transporters that confer resistance to natural product cytotoxic drugs. We recently described the complete coding sequences of four human MRP/cMOAT subfamily members and found that, among these proteins, MRP3/MOAT-D is most closely related to MRP1 (58% identity; M. G. Belinsky and G. D. Kruh, Br. J. Cancer, *80:* 1342–1349, 1999). In the present study, we sought to determine whether MRP3 is capable of conferring resistance to cytotoxic drugs. To address this question, human embryonic kidney 293 cells were transfected with an MRP3 expression vector, and the drug resistance phenotype of the transfected cells was analyzed. The MRP3-transfected cells displayed ~4-fold resistance to etoposide and ~2-fold resistance to vincristine, compared with control transfected cells. In addition, ~1.7-fold resistance was observed for the antimetabolite methotrexate. Increased resistance was not observed for several other natural product agents, including anthracyclines and Taxol. The MRP-transfected cells exhibited reduced accumulation of radiolabeled etoposide, consistent with the operation of a plasma membrane efflux pump. These results indicate that MRP3 confers resistance to some anticancer agents but that its resistance pattern is distinct from the resistance patterns of other ABC transporters involved in resistance to natural product chemotherapeutic agents.

INTRODUCTION

Cellular resistance is a major obstacle to the successful treatment of cancer with chemotherapeutic drugs. One well-established resistance mechanism involves expression of ABC[3] transporters that function to reduce intracellular drug concentrations. Pgp has served as a paradigm both for the role of efflux pumps in cellular resistance and for the development of the idea that anticancer chemotherapeutic treatments might be improved by the inclusion of modulators designed to inhibit pumps (1, 2). Because Pgp confers resistance to a spectrum of natural product drugs, including anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, and taxanes, the phenotype associated with its expression has been termed multidrug resistance. Recently, organic anion transporters have been linked to cytotoxic drug resistance. MRP1, the cDNA that encodes the M_r 190,000 protein originally identified in studies of a non-Pgp-expressing anthracycline-resistant cell line (3), has been isolated (4) and established in transfection studies as being capable of conferring a multidrug resistant phenotype (5, 6). However, in contrast to Pgp, MRP1 functions as a transporter of conjugated organic anions such as leukotriene C4 (7–9). A related organic anion transporter, cMOAT/MRP2 (10–12) has also been implicated in cytotoxic drug resistance. Transfection experiments indicate that cMOAT confers resistance to anthracyclines, *Vinca* alkaloids, etoposide, and cisplatin (13, 14). In addition, overexpression of cMOAT/MRP2 has been detected in cisplatin-resistant cell lines (10, 15).

Analyses of expressed sequence tags sequences and partial sequences have indicated that there are at least four additional human MRP/cMOAT subfamily members (15–18). The complete coding sequences and predicted structures of these four transporters, which we designated as MOAT-B (MRP4), MOAT-C (MRP5), MOAT-D (MRP3), and MOAT-E (MRP6), have recently been reported by our laboratory (19–21), and the complete sequences of the human and rat MOAT-D/MRP3 and MOAT-E/MRP6 proteins have also been reported by others (22–25). Analysis of the amino acid sequences of these four transporters indicated that they reside within an evolutionary cluster of ABC transporters that includes MRP1, cMOAT/MRP2, the cystic fibrosis transmembrane conductance regulator and the sulfonylurea receptor (19). We found that among these transporters, MOAT-D/MRP3, which we will refer to as MRP3, is the closest relative of MRP1, with which it shares 58% overall amino acid identity as well as a striking 71 and 74% identity in its first and second nucleotide binding folds, respectively. In addition, like MRP1, MRP3 possesses three membrane-spanning domains, a distinctive structural feature that is also found in cMOAT and MOAT-E/MRP6 but not in MOAT-B/MRP4 and MOAT-C/MRP5. In contrast to MRP1, which is widely expressed (26), and cMOAT/MRP2, whose expression is largely restricted to the liver, MRP3 transcript expression is moderately restricted, with abundant mRNA levels detected in the kidney, liver, colon, and pancreas (15, 20, 22, 23). In the present study, we examined whether MRP3 confers resistance to anticancer agents.

MATERIALS AND METHODS

Vector Construction and Transfection. The MRP3 cDNA (20) was assembled in Bluescript SK (Stratagene, La Jolla, CA). The nucleotides preceding the ATG initiation site were modified to CACC**ATG** using PCR, to better conform to the Kozak consensus sequence. The fidelity of the coding sequence was confirmed by nucleotide sequence analysis. The MRP3 cDNA fragment was inserted into the pcDNA3.1 eukaryotic expression vector (Invitrogen, Carlsbad, CA) to create pcDNA3-MRP3. A baculovirus expression construct was prepared by inserting the MRP3 coding sequence into pVL1392 (PharMingen, San Diego, CA).

HEK 293 cells were grown in DMEM supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin, and were electroporated with 10 μg of either the pcDNA3-MRP3 or the parental pcDNA3.1 vector using a Bio-Rad Gene Pulser apparatus. At 48 h after electroporation, the growth medium was changed to include 1 mg/ml G418, resistance to which is conferred by the neomycin resistance gene of pcDNA3.1. At ~3 weeks, independent G418-resistant colonies were isolated using the cloning cylinder technique and were expanded for immunoblot analysis. Generation of MRP3 baculovirus and infection of Sf9 cells were performed according to the manufacturer's directions (PharMingen).

Generation of MRP3 Antibody and Immunoblotting. A cDNA fragment encoding amino acids 1259–1308 of MRP3 was inserted downstream of the

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[3] The abbreviations used are: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; cMOAT, canalicular multispecific organic anion transporter; HEK, human embryonic kidney; MRP, multidrug resistance-associated protein; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; Pgp, P-glycoprotein.

glutathione *S*-transferase coding sequence in the PGEX2T prokaryotic expression vector (Pharmacia, Piscataway, NJ). The resulting fusion protein was induced in bacterial cultures and was purified using glutathione beads according to the manufacturer's recommendations. Rabbits were immunized with the purified recombinant protein, and the specificity of the resulting antiserum was confirmed in immunoblots of lysates prepared from insect cells expressing the full-length MRP3 protein.

For preparation of crude membrane fractions, HEK 293 cells were collected by incubation at 37°C for 5 min in Cell Dissociation Solution (Sigma Chemical Co., St. Louis, MO) and harvested by centrifugation at 4°C. Cell pellets were resuspended in sucrose-EDTA buffer [250 mM sucrose, 1 mM EDTA (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin]. Cell lysates were homogenized using a Dounce homogenizer and centrifuged at 3,000 $\times$ *g* for 10 min at 4°C. The supernatant was then centrifuged at 100,000 $\times$ *g* for 45 min at 4°C. The pellets were suspended in a small amount of sucrose-EDTA buffer, and an equal volume of 2$\times$ SDS sample buffer was added. Crude cell lysates of Sf9 cells were prepared using Insect Cell Lysis Buffer (PharMingen) according to the manufacturer's directions. Protein samples (100 μg) were analyzed by SDS PAGE and immunoblotting using anti-MRP3 antibody at a dilution of 1:1000.

Analysis of Drug Sensitivity and Etoposide Accumulation. Drug sensitivity was analyzed using a tetrazolium salt microtiter plate assay (CellTiter 96 Cell Proliferation Assay, Promega, Madison, WI). Cells were seeded in triplicate at 8000/well in 96-well dishes in complete medium supplemented with 10% fetal bovine serum. The next day drugs at various dilutions were added to the growth medium. Assays were performed after 72 h of growth in the presence of drug. For etoposide accumulation experiments, cells (2.5×10^6/ml) were incubated at 37°C with [^{3}H]etoposide (Moravek, Brea, CA) at a concentration of 0.2 μM. Aliquots (1.0 ml) of cells were removed at various time points and immediately added to 10 ml of ice-cold PBS. The cells were pelleted at 4°C and washed twice with 10 ml of ice-cold PBS. The cells were lysed in 1% SDS, and radioactivity was measured in a liquid scintillation counter.

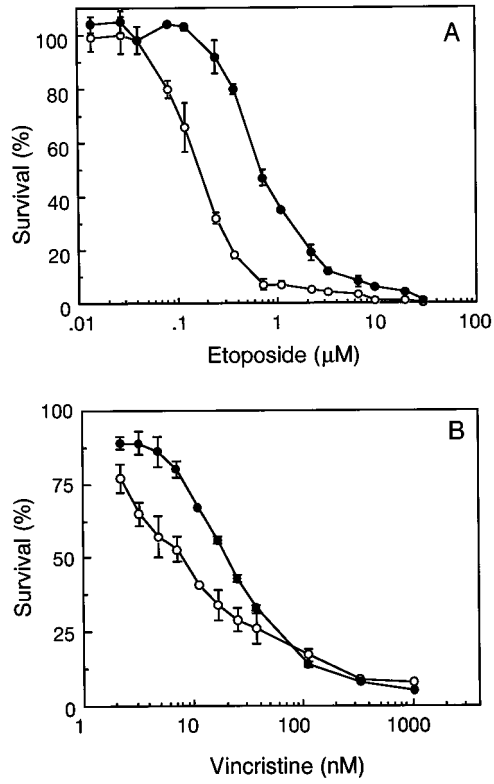

Fig. 2. Sensitivity of MRP3-transfected HEK 293 cells to etoposide (*A*) and vincristine (*B*). The drug sensitivity of parental vector-transfected cells (○) or HEK/MRP3–5 cells (●) was analyzed using the MTS/phenazine methosulfate assay as described in the "Materials and Methods." *Data points*, the mean of triplicate determinations in a single experiment.

RESULTS

The integrity of the MRP3 coding sequence was first tested by expressing the recombinant protein in insect cells. Fig. 1 shows an immunoblot analysis of MRP3 expressed in Sf9 cells *(Lane 1)*. MRP3 expressed in Sf9 cells migrated with an apparent molecular weight of M_r ~171,000, quite close to the predicted molecular mass of M_r 168,000. Having established the integrity of the coding sequence and the ability of the anti-MRP3 antibody to recognize the recombinant protein, we next sought to overexpress MRP3 in cultured cells by transfection. We reasoned that expression of MRP3 in a cell line that is derived from an organ in which the transporter is normally expressed might enhance the opportunity for proper subcellular localization and function. HEK 293 cells were, therefore, selected as the recipient cell line. HEK 293 cells were electroporated with either the pcDNA3-MRP3 expression vector described in the "Materials and Methods," or the parental pcDNA3.1 vector. Colonies were selected for growth in the presence of G418, resistance to which is conferred by the aminoglycoside 3′ phosphotransferase gene of the pcDNA3.1 vector. Membranes were prepared from G418-resistant colonies and examined for expression of MRP3 by immunoblot analysis. Increased MRP3 expression relative to parental vector-transfected cells was detected in a few colonies. One of these colonies, HEK/MRP3–5, in which MRP3 was well expressed, was selected for detailed characterization. Detection of MRP3 expressed in HEK/MRP3–5 is shown in Fig. 1 (*Lane 4*). The protein migrated predominately as two bands of apparent molecular weights M_r ~192,000 and ~171,000. The smaller of these two bands comigrated with MRP3 expressed in insect cells, which are glycosylation-deficient, which suggests that the doublet observed in HEK 293 cells represents differentially glycosylated forms of the protein.

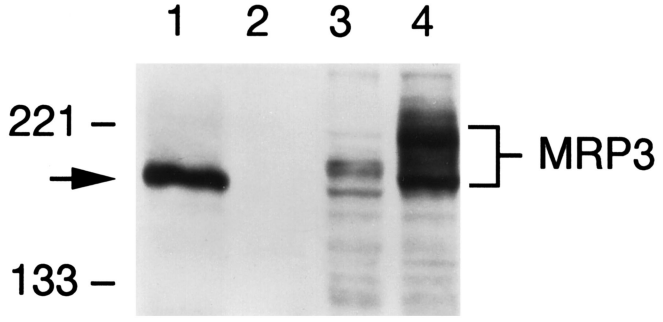

Fig. 1. Immunoblot detection of MRP3 in transfected HEK 293 cells. Cellular lysates of Sf9 cells (100 μg) or membrane preparations of HEK 293 cells (100 μg) were separated by SDS-PAGE, and MRP3 was detected by immunoblotting with polyclonal anti-MRP3 antibody. *Lane 1*, MRP3 expressed in Sf9 cells; *Lane 2*, Sf9 cells infected with a control baculovirus; *Lane 3*; HEK 293 cells transfected with the parental pcDNA3.1 plasmid; *Lane 4*; HEK/MRP3–5 cells. The locations of protein molecular weight markers are shown *to the left*. *Arrow*, MRP3 expressed in Sf9 cells.

The drug-resistance phenotype of HEK/MRP3–5 was analyzed to determine the influence of MRP3 overexpression on drug sensitivity. As shown in Fig. 2, the MRP3-transfected cells displayed an ~ 4-fold increased resistance to the natural product drug etoposide. In addition, increased resistance was also detected for vincristine, to which HEK/MRP3–5 cells were ~2-fold resistant. Resistance to these two agents was confirmed in a second transfectant in which MRP3 was expressed at slightly lower levels compared with HEK/MRP3–5. This transfectant exhibited ~3- and ~1.5-fold resistance to etoposide and vincristine, respectively (data not shown). As summarized in Table 1, increased resistance was not observed for several other lipophilic cytotoxic drugs, including anthracyclines, mitoxantrone, and Taxol, as

Table 1 *Drug sensitivity of HEK/MRP3-5 cells*

Drug	IC_{50}[a] (nM) HEK/pcDNA3.1	HEK/MRP3–5	N[b]	Relative resistance[c]
Etoposide	147 ± 47	631 ± 219	11	4.29[d]
Vincristine	11.6 ± 3.46	26.0 ± 7.25	10	2.24[d]
Doxorubicin	28.0 ± 15	24.0 ± 11	4	0.87
Daunorubicin	18.2 ± 5.9	19.8 ± 3.4	5	1.09
Taxol	27.8 ± 11.9	29.2 ± 11.4	5	1.05
Actinomycin D	2.63 ± 0.34	2.95 ± 0.30	6	1.12
Mitoxantrone	11.0 ± 2.0	10.0 ± 1.4	4	0.91
Estramustin	4480 ± 670	4920 ± 790	6	1.10
Methotrexate	22.0 ± 5.0	38.3 ± 12.8	11	1.74[d]
Cisplatin	2050 ± 420	2400 ± 520	4	1.17

[a] Drug concentration that inhibited cell survival by 50%.
[b] Number of independent experiments, each performed in triplicate.
[c] IC_{50} of HEK/MRP3–5 transfectant ÷ IC_{50} of control transfectant.
[d] Significantly different from control transfectant as assessed by Student's t test ($P < 0.001$).

well as for the alkylating agent cisplatin. However, low (~1.7-fold) but statistically significant resistance was observed for the antimetabolite methotrexate.

The high degree of structural similarity between MRP1 and MRP3 suggests that MRP3 may confer resistance by functioning to extrude drugs from the cell. To examine the cellular-resistance mechanism associated with MRP3 expression, the accumulation of radiolabeled etoposide was, therefore, examined. As shown in Fig. 3, HEK/MRP3–5 exhibited an accumulation deficit compared with the control transfected cells. At 1 h after incubation with radiolabeled etoposide, accumulation in HEK/MRP3–5 cells was 73% of the accumulation observed in the control transfected cells. Reduced accumulation was also observed in a second transfectant (data not shown). Accumulation experiments were not performed with vincristine or methotrexate because of the low levels of resistance observed with these agents.

DISCUSSION

This study indicates that expression of MRP3, the closest known relative of MRP1, confers resistance to several anticancer agents. MRP3, therefore, joins four other ABC transporters that also confer resistance to natural product agents: Pgp, MRP1, cMOAT/MRP2, and BCRP/MXR. Interestingly, each of these transporters confers an overlapping but distinct resistance pattern. The Pgp phenotype is the broadest with regard to natural product agents and includes anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, mitoxantrone, actinomycin D, and Taxol (1, 2). The MRP1 (27–29) and cMOAT/MRP2 (14, 30) phenotypes are similar to Pgp but do not include Taxol and, in the case of MRP1, mitoxantrone.[4] In addition, MRP1 and MRP2/cMOAT have been reported to confer resistance to camptothecins (13, 30). Our results indicate that MRP3 confers resistance to etoposide and vincristine but does not confer resistance to Taxol, anthracyclines, and mitoxantrone. The recently described BCRP/MXR transporter (31–33) has been reported to confer resistance to anthracyclines and mitoxantrone but not to Taxol and etoposide (31). In addition, some of these ABC transporters have recently been implicated in resistance to classes of chemotherapeutic agents other than natural products. MRP1 and MRP2/cMOAT have been reported to confer resistance to methotrexate (34), an activity that is consistent with the previous observation that MRP2/cMOAT plays a role in the hepatobiliary excretion of this agent in rats (35), and MRP2/cMOAT has been reported to confer resistance to the alkylating agent cisplatin (13, 14). Our results indicate that methotrexate is also part of the MRP3 profile. The growing number of efflux pumps with overlapping resistance profiles, and the expression of some of these pumps in excretory organs (*e.g.*, Pgp, MRP2/cMOAT, MRP3), may pose significant challenges to strategies designed to improve clinical outcomes by inhibiting plasma membrane transporters, both in terms of redundant activities and pharmacokinetic perturbations. However, the extended profiles of some of these transporters with regard to nonnatural product agents may present unexpected opportunities for clinical modulation strategies in that natural product agents are often combined with cisplatin and/or methotrexate in many commonly used chemotherapeutic regimens. An inhibitor might, therefore, simultaneously enhance the sensitivities of both the natural product and the nonnatural product components of a multidrug regimen. The balance of these opposing considerations should be revealed as clinical trials of inhibitors proceed.

Decreased accumulation of etoposide in MRP3-transfected cells supports the idea that it contributes to resistance by functioning as a plasma membrane efflux pump to reduce intracellular drug concentrations. Although we have not localized MRP3 in our transfectants, the reported detection of human MRP3 in hepatocyte basolateral membranes supports plasma membrane localization (36). It is also possible that MRP3 contributes to resistance by increasing cytoplasmic sequestration of drug in export vesicles, a feature we have observed for MRP1-conferred resistance (27). The biochemical mechanism that underlies MRP3-conferred resistance remains to be established. The high degree of amino acid identity between MRP3, MRP1 and MRP2/cMOAT suggests the possibility that, like the latter transporters, MRP3 may function as an organic anion transporter. This possibility is suggested by a recent report concerning the rat homologue of MRP3, for which transport of the glucuronide 17β estradiol-17β-D-glucuronide and methotrexate were detected in membrane vesicle assays (37). However, the transport of glutathione conjugates such as leukotriene C4, which are excellent substrates for MRP1, was not detected. Although the transport of methotrexate by the rat homologue is consistent with our observation that MRP3 confers resistance to this agent, the absence of detectable transport of glutathione conjugates does not seem to support a model in which MRP3 cotransports natural product agents with glutathione, as has been proposed for MRP1 (38, 39). However, the substrate specificity of the human MRP3 protein has not yet been established in biochemical transport studies, and it is possible that the substrate specificities of the human and rat proteins may differ. Additional experiments with the recombinant human MRP3 protein should define its substrate specificity and help to elucidate the biochemical mechanism whereby it confers resistance to natural product agents.

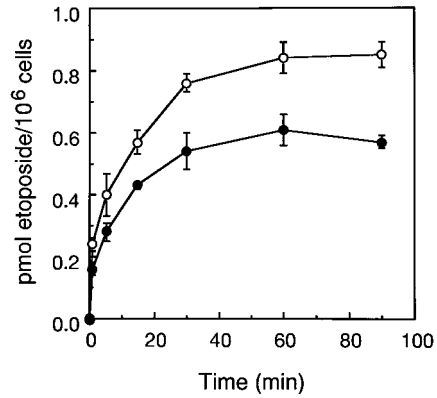

Fig. 3. Accumulation of radiolabeled etoposide in MRP3-transfected HEK 293 cells. Control pcDNA3-transfected (○) or HEK/MRP3–5 (●) cells were incubated in the presence of 0.2 μM [^{3}H]etoposide, and the accumulation was measured at various time points. *Data points*, the mean of triplicate determinations in a single experiment. This experiment was repeated four times with similar results.

[4] The sensitivity of cMOAT-transfected cells to mitoxantrone and several other drugs has not been reported.

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