Comparative Study of the Importance of Multidrug Resistance-associated Protein 1 and P-Glycoprotein to Drug Sensitivity in Immortalized Mouse Embryonic Fibroblasts[1]

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Abstract

Multidrug resistance-associated protein 1 and P-glycoprotein are major ATP-binding cassette transporters that function as efflux pumps and confer resistance to a variety of structurally unrelated anticancer agents. To evaluate the comparative importance of these transporters with respect to anticancer agents, we established and characterized SV40-immortalized [*mrp1*(−/−)] (KO), [*mdr1a*/*1b*(−/−)] (DKO), and combined [*mrp1* (−/−), *mdr1a*/*1b*(−/−)] (TKO) deficient fibroblast lines derived from primary embryonic fibroblasts of knockout mice. Western blot analyses demonstrated that KO and DKO fibroblasts exhibited similar levels of P-glycoprotein and mrp1, respectively, to that of wild-type (WT) fibroblasts. In addition, semiquantitative reverse transcription-PCR measurements of other multidrug resistance-associated protein (mrp) family members demonstrated that TKO fibroblasts displayed expression profiles of mrps 2–7 comparable to that of WT fibroblasts. These results indicate that loss of mrp1, P-glycoprotein, or both transporters does not cause overt compensatory changes in the expression of the other determined transporters. Using cell viability and calcein accumulation assays, we demonstrated that KO and DKO fibroblasts exhibited a low to moderate increase in sensitivity to vincristine and etoposide and in calcein accumulation compared to WT fibroblasts, whereas TKO fibroblasts displayed a markedly enhanced sensitivity to these agents and further elevated calcein accumulation. Furthermore, verapamil, an inhibitor of both mrp1 and P-glycoprotein, significantly sensitized WT fibroblasts to both vincristine and etoposide while having no effect on the sensitivity of TKO cells to these agents. Collectively, these findings indicate that mrp1 and P-glycoprotein are major determinants of drug sensitivity in immortalized mouse embryonic fibroblasts. They also suggest the existence of a compensatory mechanism by which the loss of one transporter can be functionally offset by the other in the transport of common drug substrates.

Introduction

The M_r 170,000 P-glycoprotein, encoded by the *MDR*[3]*1* gene, and the M_r 190,000 MDR-associated protein 1 (MRP1) are the two best characterized ABC transporters involved in resistance to a variety of structurally unrelated anticancer agents of natural origins, including the *Vinca* alkaloids, anthracyclines, and epipodophyllotoxins. Both P-glycoprotein and MRP1 are membrane-associated, energy-dependent efflux pumps that function to reduce the intracellular levels of hydrophobic cytotoxic compounds (1–3). Despite similarities in drug resistance profiles, these transporters differ markedly in their substrate selectivities. MRP1 is capable of transporting many lipophilic anions, including conjugates of glutathione, glucuronic acid, and sulfate (4–6). MRP1 also mediates the cotransport of unmodified anticancer drugs with free glutathione (7, 8). In contrast, P-glycoprotein predominately transports neutral or mildly cationic molecules. Neither conjugation nor cotransport of drug substrates with physiological molecules is required for the efflux process mediated by P-glycoprotein.

In addition to MRP1, eight other MRPs have been identified to date, including MRP2 ($ABCC_2$, cMOAT), MRP3 ($ABCC_3$, MOAT-D), MRP4 ($ABCC_4$, MOAT-B), MRP5 ($ABCC_5$, MOAT-C), MRP6 ($ABCC_6$, MOAT-E), MRP7 (ABCC10), MRP8 (ABCC11), and MRP9 (ABCC12). Comparison of the predicted structures of the MRPs reveals that MRP1, MRP2, MRP3, MRP6, and MRP7 all consist of a NH_2-terminal domain (TMD_0) in addition to a cytoplasmic linker (L_0), and a MDR1-like core structure (9–11). In contrast, MRP4, MRP5, MRP8, and MRP9 lack the TMD_0 domain but possess the L_0 segment (11–15). Compared to MRP1, MRP4, MRP5, MRP8, and MRP9, which are widely expressed in tissues (11–15), MRP2, MRP3, and MRP6 are predominately expressed in liver and kidney, with MRP3 also expressed in intestine, pancreas, and a few other tissues (11, 16–18). Similar to MRP1, MRP2 is able to confer

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[3] The abbreviations used are: MDR, multidrug resistance; Mrp, MDR-associated protein; ABC, ATP-binding cassette; WT, wild-type; KO, *mrp1*(−/−) gene deficiency; DKO, *mdr1a*/*1b*(−/−) gene deficiency; TKO, combined *mrp1*(−/−), *mdr1a*/*1b*(−/−) gene deficiency; RT-PCR, reverse transcription-PCR; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; calcein AM, calcein acetoxymethyl ester.

resistance to a variety of anticancer drugs, with additional resistance to cisplatin (19–21). The drug resistance profile of MRP3 is limited to a few anticancer drugs, including etoposide, vincristine, and methotrexate (22, 23). MRP4 has been shown to confer resistance to methotrexate, 6-thiopurines, and the antiviral purine analogue 9-(2-phosphonylmethoxyethyl)adenine (24–26). MRP5 is also capable of conferring resistance to 6-thiopurines and antiviral nucleoside analogues, but not to most other anticancer drugs (27, 28). The involvement of MRP6 in drug resistance is currently unclear. MRP7 closely resembles MRP1, MRP2, MRP3, and MRP6 structurally but has the lowest homology to other MRP members (29). The abilities of MRP7, MRP8, and MRP9 to efflux anticancer drugs remain to be determined.

To better understand the contributions of mrp1 and P-glycoprotein to anticancer drug sensitivity, we have previously examined the pharmacological consequences of *mrp1*-, *mdr1a/1b*-, and combined *mrp1* and *mdr1a/1b*-deficiencies in knockout mice (30). Our findings demonstrated that mice with combined *mrp1*(−/−), *mdr1a/1b*(−/−) deficiencies exhibited enhanced sensitivities of 128-fold to vincristine and 3.5-fold to etoposide over that of WT mice. *In vitro*, enhanced sensitivities of primary embryonic fibroblasts developed from the combined *mrp1*(−/−), *mdr1a/1b*(−/−) mice were also observed with these drugs. The results collectively indicate that increased drug sensitivity is attributable to deficiencies of multiple transporters and that P-glycoprotein and mrp1 are compensatory efflux pumps for vincristine and etoposide.

In this study, the relative importance of the P-glycoprotein and mrp1 to drug sensitivity was further elucidated by using immortalized fibroblast cell lines developed from WT; *mrp1*(−/−); *mdr1a/1b*(−/−); and *mrp1*(−/−), *mdr1a/1b*(−/−) primary embryonic fibroblasts. These cell lines were characterized with regard to the expression profiles of mrp family members, their sensitivities toward a panel of anticancer drugs, the accumulation of the fluorescent dye calcein, and the effects of an established MDR pump inhibitor. The results provide additional evidence that both mrp1 or P-glycoprotein can function to compensate for the loss of the other in the transport of common drug substrates without an increase in the amount of transporter and underscore the predominant role of these two transporters with respect to the drug sensitivity of normal and possibly neoplastic cells.

Materials and Methods

Cells and Chemicals. WT, KO, DKO, and TKO mouse primary embryonic fibroblasts were prepared from 13–14 day-old embryos and cultured as described previously (30). Immortalized fibroblasts derived from primary embryonic fibroblast lines were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin antibiotics. The plasmid pCC2pA that contains the entire SV40 genome (31) was kindly provided by Dr. Charles N. Cole (Dartmouth Medical School, Hanover, NH). Vincristine, vinblastine, etoposide, paclitaxel, methotrexate, doxorubicin, mitomycin C, and MK-571 were purchased from Biomol (Plymouth Meeting, PA). PGP-4008 was purchased from Alexis (San Diego, CA). Cisplatin and verapamil were obtained from Sigma (St. Louis, MO). Calcein and calcein AM were purchased from Molecular Probes (Eugene, OR).

Transfection and Immortalization of Primary Fibroblasts. Immortalization of primary embryonic fibroblasts was performed as described by Conzen and Cole (32). WT, KO, DKO, and TKO primary embryonic fibroblasts (1.5×10^5 cells) were plated in 100-mm tissue culture dishes for 24 h before transfection. Cells were cotransfected with 15 μg of pCC2pA and 1.5 μg of pCMV/Bsd (Invitrogen, Carlsbad, CA) using lipid-mediated transfection (TransFast Reagent; Promega, Madison, WI) according to the manufacture's instructions. Three μg/ml of blasticidin S were added to the medium at 72 h after transfection to select for cells that coexpressed the blasticidin S resistance protein and the SV40 large T antigen. After 11–12 days, when blasticidin S-resistant colonies became evident, individual colonies were pooled and expanded into cell lines. Immortalized fibroblasts have been grown continuously in culture for >30 passage generations without any sign of growth cessation.

Immunoprecipitation and Western Blotting. Cells were collected and dissolved in immunoprecipitation buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.5% NP40] containing a mixture of protease inhibitors (Roche, Indianapolis, IN). The lysate supernatant was incubated with mouse monoclonal anti-SV40 large T antigen antibody (Chemicon, Temecula, CA) at 4°C for 1 h and then incubated with Protein A/G Plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) with constant agitation at 4°C overnight. The immunoprecipitate was collected, washed, and resuspended in 1× SDS sample buffer [125 mM Tris-HCl (pH 6.8), 5% glycerol, 2% SDS, 0.006% bromphenol blue, and 1% β-mercaptoethanol] and boiled for 5 min. For proteins directly analyzed by Western blotting, the cell pellets were lysed with lysis buffer [10 mM Tris-HCl (pH 7.4) and 1% SDS), boiled for 5 min, and passed through a needle several times to shear DNA. Protein concentrations were determined by the Bio-Rad D_C protein assay (modified Lowry method; Bio-Rad, Hercules, CA) according to the manufacture's instructions. Forty μg of protein were separated by electrophoresis on a 10% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% milk in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4°C overnight. The membrane was subsequently washed with TBST, incubated with the horseradish peroxidase-conjugated secondary antibody in blocking solution at room temperature for 1 h, and washed again. The protein band was visualized by the enhanced chemiluminescent method (NEN, Boston, MA).

RNA Isolation and RT-PCR Analyses. mRNA was extracted using the Fast Track 2.0 mRNA purification kit (Invitrogen) according to the manufacture's protocol. Cell pellets ($4–5 \times 10^7$ cells) and pulverized powdered tissues (0.7–1 g) were dissolved in 15 ml of stock/lysis buffer containing 300 μl of protein/RNase degrader. The lysate was then incubated with up to 150 mg of oligo(dT)-cellulose at 60 min at room temperature. Oligo(dT)-cellulose was washed with low salt buffer and transferred to a spin-column/

Table 1 The primer sequences of mrps 1–7 for RT-PCR and expected sizes of PCR products

	Forward primers (5′→3′)	Reverse primers (5′→3′)	Sizes of PCR product (bp)
Mrp1	GTTCCCTCCGCATGAACTTG	CTGGCTCATGCCTGGACTCTG	550
Mrp2	TGAACTGGCTAGTGAGGA	GTCAGTCTCCCTCGAAGG	380
Mrp3	ACCATCATGGTCATCGTG	CATGGTCACCTGTAAGGCA	420
Mrp4	GTTCACTGGAACCATGAG	TGTGAGCAATGGTGAGCA	320
Mrp5	AGTAATGGAGAACGGGGA	ACTTCGAGGACGGGAGGA	370
Mrp6	ATTAGCGATGCCTTCAGG	AAGAGCAGCCACAGCCCGTTG	340
Mrp7	ACTGGCTGTCTCAGCTGA	AAGCCCTGTAGTAGCCCT	550

microcentrifuge set. mRNA was eluted, precipitated, and dissolved in 25–50 μl of water. mRNA concentration was quantified by spectrophotometry.

RT-PCR of mrps 1–7 and β-actin was performed using the Access RT-PCR System (Promega) in 50-μl reactions using 0.2 μg of mRNA as template. The final concentrations of the reagents in each 50-μl reaction mix were as follows: 1× avian myeloblastosis virus/*Tfl*, 5× reaction buffer, 0.2 mM deoxynucleoside triphosphate mix, 1 mM $MgSO_4$, 0.1 units/μl of *Tfl* DNA polymerase, 0.2 μM forward and reverse primer, and 0.1 units/μl of avian myeloblastosis virus reverse transcriptase. A kit control reaction was run with each group of reactions, as were negative control reactions in which reverse transcriptase was deleted. The mouse β-actin primer set (Stratagene, La Jolla, CA) sequences were as follows: (5′-3′) sense TGTGATGGTGGGAATGGGTCAG; antisense TTTGATGTCACGCACGATTTCC. The sequences of the primers for mrps 1–7 and expected sizes of PCR product are listed in Table 1. The primer sequences for mrp1 amplification were previously reported (33), and the primer pairs for mrp2 (AF227274), mrp5 (AB019003), and mrp6 (AB028737) were based on corresponding GenBank entries. The sequence data used to generate primer pairs for mrp3, mrp4, and mrp7 were based on clones of mouse mrp3, mrp4, and mrp7 cDNAs.[4] The RT-PCR conditions were one cycle of 48°C for 45 min and 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, 68°C for 2 min, and finally an extension cycle of 68°C for 7 min. PCR product was separated on a 1.5% agarose gel containing ethidium bromide. The images were scanned and analyzed using a Bio-Rad gel documentation system.

Drug Sensitivity Assay. Immortalized fibroblasts were seeded into 96-well plates at 3000 cells/well in complete DMEM and incubated for 24 h. Each drug was then added in various concentrations to quadruplicate wells in a final volume of 100 μl of medium. After 72 h of incubation, 20 μl of MTS tetrazolium reagent (CellTiter 96 One solution; Promega) were added, and the incubation was continued for an additional 2 h at 37°C. The absorbance of soluble MTS tetrazolium formazan produced by viable cells after drug exposure was measured at 490 nm using a microplate reader. The sensitivity of fibroblasts to each drug was expressed as a percentage of the vehicle-treated control. For verapamil pretreatment, cells were first exposed to 10 μM verapamil for 1 h; vincristine and etoposide were added thereafter in various concentrations to quadruplicate wells in a final volume of 100 μl of medium. After 72 h of incubation, sensitivities were determined by the MTS assay as described above. All data are means of at least three independent experiments.

Intracellular Accumulation of Calcein. Immortalized fibroblasts were plated onto 6-well plates at a density of 1.0–1.5 × 10^5 cells/well and grown for 48 h. Calcein AM (Molecular Probes) was added in various concentrations to wells containing 3 ml of the culture medium. After 1-h exposure of calcein AM, the medium was removed by aspiration, and cells were washed three times with ice-cold PBS. The cellular monolayer was immediately solubilized with 500 μl of 1% Triton X-100 in PBS. One hundred μl of lysate were used for determination of calcein fluorescence at excitation/emission wavelengths of 494/517 nm using a fluorescence microplate reader. The protein concentration of the lysate was determined by the Bio-Rad D_C protein assay. The intracellular concentration of calcein was quantified against a standard curve of calcein and expressed as nanomoles of calcein/mg of cellular protein.

Results

Immortalization of Mouse Embryonic Fibroblasts. We have previously isolated and cultured primary embryonic fibroblasts derived from 13–14 day-old embryos of WT, KO, and TKO mice established in our laboratory (30) and of DKO mice [developed by Schinkel *et al.* (34)] purchased from Taconic Farms, Inc. (Germantown, NY). However, the use of these knockout cells in our studies was limited by the fact that primary cells typically exhibit a short life span in culture (35). To overcome this limitation, we have established immortalized cell lines from these primary embryonic fibroblasts by transfection with the SV40 genome. Five to 10 immortalized clones of each genotype were established. These fibroblast lines grew continuously in culture and did not exhibit any sign of growth cessation with time, as did their nonimmortalized counterparts. One clone from each genotype with similar doubling times was chosen for subsequent experiments. The doubling times of the chosen WT, KO, DKO, and TKO immortalized fibroblast lines were 24, 21, 20, and 23 h, respectively.

Immortalization of primary rodent cells by the SV40 viral DNA has been shown to correlate with the ability of the encoded large T antigen to associate with the tumor suppressor protein p53 and the retinoblastoma protein Rb (31). To ensure that established fibroblast lines were immortalized through transfection with the SV40 genome, expression of

[4] M. G. Belinsky and G. D. Kruh, unpublished sequences.

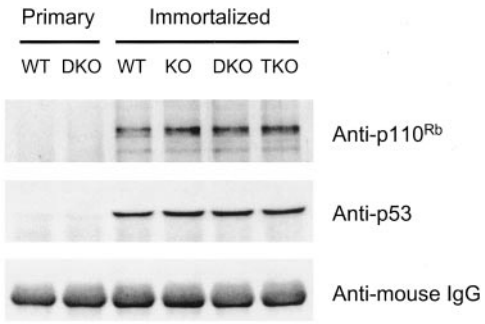

Fig. 1. Immunoprecipitation of cellular proteins associated with the SV40 large T antigen in mouse embryonic fibroblasts. Total protein from primary and immortalized WT, KO, DKO, and TKO fibroblast cell lines was immunoprecipitated with mouse anti-SV40 large T antigen antibody. Cellular proteins coprecipitated with large T antigen were analyzed by Western blotting using goat anti-p53 and rabbit anti-Rb antibodies. Antimouse IgG antibody was used to show approximately equal loading of each lane.

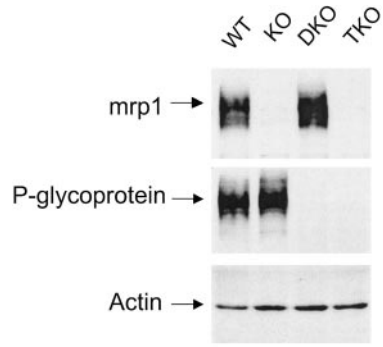

Fig. 2. Western blot analyses of mrp1 and mdr1a/1b expression in immortalized fibroblast lines. Total protein from WT, KO, DKO, and TKO fibroblast lines was analyzed by Western blotting using rat anti-MRP1 (MRPr1) and mouse anti-mdr1a/1b P-glycoprotein (C219) antibodies. Blots were also probed with anti-actin antibody to show approximately equal loading of each lane.

the large T antigen in fibroblasts was examined indirectly by immunoprecipitation of the large T antigen followed by Western blot analysis of its associated cellular proteins. The large T antigen was immunoprecipitated using a mouse monoclonal antibody raised against the SV40 large T antigen. The immunocomplex was analyzed thereafter by Western blotting using anti-p53 and anti-Rb antibodies (Santa Cruz Biotechnology). Fig. 1 shows that both p53 and Rb proteins were detected only in immortalized WT, KO, DKO, and TKO fibroblasts but not in primary WT and DKO fibroblasts used as negative controls. Because spontaneous immortalization of rodent primary cells frequently occurs, this result demonstrates that these fibroblasts were immortalized as a result of expression of the SV40 large T antigen and its association with the cellular proteins p53 and Rb.

To examine the expression patterns of mrp1 and mdr1a/1b, Western blot analyses were performed with each of the four fibroblast cell lines using anti-MRP1 (MRPr1) and anti-P-glycoprotein (C219) antibodies (Signet, Dedham, MA). Fig. 2 shows that mrp1 protein was expressed in WT and DKO fibroblasts and that the mdr1a/1b proteins were expressed in WT and KO fibroblasts. As expected, both mrp1 and mdr1a/1b proteins were absent in TKO fibroblasts. There was no obvious increase in the expression of P-glycoprotein in KO fibroblasts, nor increase in the expression of mrp1 in DKO fibroblasts.

Determination of the Expression of mrps 1–7 in Immortalized Fibroblasts. To determine whether the loss of both mrp1 and mdr1a/1b proteins cause changes in the expression pattern of other mrp family members, RT-PCR analyses of mrps 1–7 were performed with immortalized WT and TKO fibroblasts. In addition, mRNAs from WT mouse liver and lung were used as positive controls to confirm the specificity of the selected PCR primers. mRNAs were amplified individually using primers specific for each mrp member, and the levels of corresponding PCR products were visualized on an agarose gel (Fig. 3, *A* and *B*). The identity of the PCR products for all of the mrp members was verified by their molecular size and by nucleotide sequencing. The results showed that mrp4 and mrp5 were ubiquitously expressed at moderate levels in the immortalized fibroblast lines and in mouse liver and lung tissue (Fig. 3, *A* and *B*). In fibroblasts, the expression of mrp2, mrp3, and mrp6 appeared to be very low or not detectable (Fig. 3*A*). Both WT and TKO fibroblast cell lines exhibited a similar expression pattern for all MRP family members, except that mrp1 PCR product was not detected in TKO fibroblasts because the mrp1 primers targeted the mrp1 disruption site (33). A modest increase in the expression of mrp3 appeared to occur in TKO fibroblasts compared with that of WT fibroblasts. In contrast to immortalized fibroblasts, mouse liver showed high expression levels of mrp2, mrp3, and mrp6 (Fig. 3*B*), whereas in the lung, the expression of mrp3 was the most prominent, and expression of mrp2 and mrp6 was not detected.

Sensitivity of Immortalized Fibroblasts to Various Anticancer Agents. The effects of the deficiencies in mrp1, P-glycoprotein, or both transporters on the sensitivity of immortalized fibroblasts to various anticancer agents were studied. After exposure of each fibroblast line to drugs for 72 h, cell viability was measured by the MTS assay. Fig. 4 demonstrates the percentage of survival of all fibroblast lines over a range of concentrations of vincristine and etoposide. The fold increase in sensitivity of KO, DKO, and TKO cells to both drugs and other anticancer agents compared with WT cells, expressed as IC_{50}s, is shown in Table 2.

Vinca alkaloids and epipodophyllotoxins are known substrates of both mrp1 and P-glycoprotein, and increases in sensitivity to these two agents were observed in knockout fibroblasts. KO, DKO, and TKO fibroblasts exhibited 1.7-, 2.5-, and 12-fold increases, respectively, in their sensitivity to vincristine. Similarly, KO, DKO, and TKO fibroblasts exhibited 3.4-, 1.3-, and 8.2-fold increases, respectively, in their sensitivity to etoposide. These findings demonstrated that deficiencies in either mrp1 or P-glycoprotein caused a low to moderate increase in sensitivity to vincristine and etoposide, whereas lack of both transporters resulted in a markedly enhanced sensitivity of immortalized fibroblasts to these agents.

The sensitivity of immortalized fibroblasts to other anticancer agents transported by either mrp1 or P-glycoprotein was also determined. Paclitaxel is a substrate for P-glycoprotein

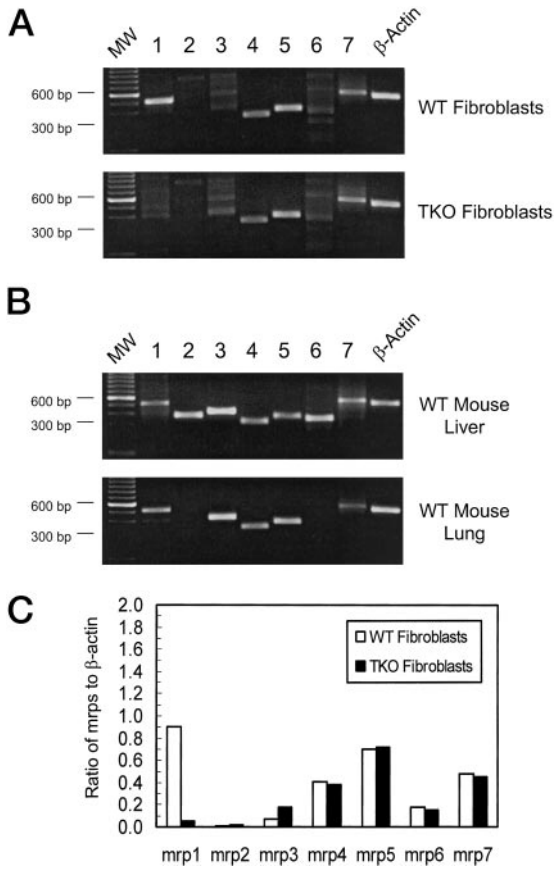

Fig. 3. RT-PCR analyses of the expression of mrps 1–7 mRNAs in WT and TKO immortalized fibroblasts. mRNAs (0.2 μg) from immortalized fibroblasts (*A*) and 5-month-old WT mouse liver and lung (*B*) were amplified individually using oligonucleotide primers specific for mouse mrps 1, 2, 3, 4, 5, 6, and 7 and β-actin. The corresponding PCR products were visualized on an agarose gel stained with ethidium bromide. *MW*, molecular size marker; *1–7*, mrps 1–7, respectively. The results are representative of two independent determinations. *C*, semiquantitative comparison of mrps 1–7 between WT and TKO immortalized fibroblasts. The band intensities of the PCR products of mrps 1–7 were quantified and normalized to that of β-actin. Values are the mean of two independent experiments.

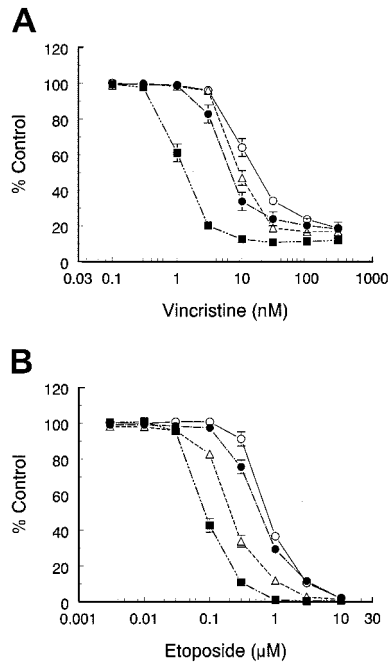

Fig. 4. Sensitivity of immortalized WT, KO, DKO, and TKO fibroblasts to vincristine (*A*) and etoposide (*B*). Immortalized WT (○), KO (△), DKO (●), and TKO (■) fibroblasts were exposed to various concentrations of drugs for 72 h. Cell viability determined by the MTS assay was expressed as a percentage of the vehicle-treated control. Data are the means ± SE (shown where larger than the symbols) of three to five independent experiments performed in quadruplicate.

but not for mrp1 (36, 37). KO fibroblasts demonstrated a 1.7-fold increase in sensitivity to paclitaxel, whereas DKO and TKO fibroblasts displayed pronounced 13.8- and 19-fold increases in their sensitivity, respectively (the paclitaxel sensitivities exhibited by DKO and TKO fibroblasts were not statistically different from each other). This result agrees with that of others (36, 37) and demonstrates that the deficiency in P-glycoprotein, but not of mrp1, is predominately responsible for the increased sensitivity of DKO and TKO fibroblasts to paclitaxel.

Methotrexate is a known substrate for mrps 1–4 (21, 23, 25). A slight (1.3-fold) increase in sensitivity to methotrexate was observed in DKO fibroblasts, whereas KO and TKO fibroblasts exhibited a similar but more pronounced sensitivity (2.8- and 3-fold, respectively). This finding supports the concept that methotrexate is a better substrate for mrp1 than for P-glycoprotein. In contrast to human MRP1, murine mrp1 does not confer resistance to doxorubicin (38, 39). In this study, KO, DKO, and TKO fibroblasts showed 1.7-, 3.5-, and 4.6-fold increases, respectively, in their sensitivity to doxorubicin. A deficiency in P-glycoprotein appeared to account for most of the increase in sensitivity to doxorubicin, whereas the lack of mrp1 produced a lesser degree of sensitization. These results support the finding of others (38, 39) demonstrating that doxorubicin is poorly transported by murine mrp1 in immortalized fibroblasts.

The sensitivity of WT and TKO fibroblasts to the alkylating agents cisplatin and mitomycin C was tested. TKO fibroblasts did not exhibit an increase in sensitivity to either cisplatin or mitomycin C, compared with WT fibroblasts. These results imply that neither of these alkylating agents is transported by mrp1 or P-glycoprotein and support the concept that differences in sensitivity to other drugs tested in this study are because of the transporter deficiencies in immortalized fibroblasts.

Intracellular Accumulation of Fluorescent Calcein and the Effects of MDR Transporter Inhibitors. Calcein AM, a substrate of MDR transporters, was used to determine the functional activities of mrp1 and P-glycoprotein in immortalized fibroblasts, as determined by the intracellular accumulation of fluorescent calcein. In addition, the effects of various inhibitors of MDR transporters on calcein accumulation in these cells were also investigated. Calcein AM is a nonfluorescent, highly lipophilic dye that readily enters cells by passive diffusion. Upon entry into cells, calcein AM is hydrolyzed by cytoplasmic esterases and converted into calcein, an

Table 2 Sensitivity of immortalized WT, KO, DKO, and TKO fibroblast cell lines to various anticancer agents

Agent	WT	KO		DKO		TKO	
	IC_{50} (nM)[a]	IC_{50} (nM)	H.F.[b]	IC_{50} (nM)	H.F.	IC_{50} (nM)	H.F.
Vincristine	18.0 ± 2.5	10.7 ± 1.1[c]	1.7	7.3 ± 0.98[c]	2.5	1.5 ± 0.14[d]	12.0
Vinblastine	17.7 ± 1.2	n.d.[e]		n.d.		3.5 ± 0.35[d]	5.1
Etoposide	767 ± 31.8	224 ± 18.1[d]	3.4	582 ± 42.2[c]	1.3	93.0 ± 8.9[d]	8.2
Paclitaxel	300 ± 41.4	180 ± 10.8[c]	1.7	21.8 ± 3.0[d]	13.8	15.8 ± 2.5[d]	19.0
Methotrexate	51.3 ± 0.75	18.8 ± 0.63[d]	2.8	39.0 ± 3.0[c]	1.3	17.5 ± 1.0[d]	3.0
Doxorubicin	117 ± 24.0	67.3 ± 3.2	1.7	33.0 ± 4.0[c]	3.5	25.7 ± 2.0[c]	4.6
Daunorubicin	110 ± 5.8	n.d.		n.d.		29.7 ± 5.2[d]	3.7
Cisplatin	2500 ± 115	n.d.		n.d.		2333 ± 88.2	0.93
Mitomycin C	253 ± 16.7	n.d.		n.d.		283 ± 8.8	1.12

[a] The IC_{50} of each agent was determined using the MTS assay. Values shown are means ± SE of three to five independent experiments.
[b] H.F., hypersensitivity factors were calculated as the ratio of the IC_{50} of WT fibroblasts to the IC_{50} of KO, DKO, and TKO fibroblasts.
[c] $P < 0.05$ (Student's *t* test), the IC_{50} of KO, DKO, or TKO fibroblasts compared with the IC_{50} of WT fibroblasts.
[d] $P < 0.001$ (Student's *t* test), the IC_{50} of KO, DKO, or TKO fibroblasts compared with the IC_{50} of WT fibroblasts.
[e] n.d., not determined.

intensely fluorescent organic anion. Calcein AM is a substrate for both mrp1 and P-glycoprotein, whereas the extrusion of hydrophilic calcein is only mediated by mrp1 (40, 41). After exposure to various concentrations of calcein AM for 1 h, a substantially increased accumulation of calcein was observed in KO and TKO fibroblasts (2.1- and 3.0-fold, respectively) compared to WT fibroblasts. The accumulation of calcein in TKO fibroblasts was significantly higher than that in KO fibroblasts (Student's *t* test; $P < 0.01$). In contrast, DKO fibroblasts exhibited a level of calcein accumulation similar to that of WT fibroblasts (Fig. 5*A*). DKO and TKO fibroblasts showed a similar increase in the accumulation of rhodamine 123, a substrate of P-glycoprotein, over that of WT and KO fibroblasts. Furthermore, no difference in rhodamine 123 accumulation occurred between WT and KO fibroblasts (data not shown).

The effects of established inhibitors of mrp1 and P-glycoprotein on the accumulation of calcein in immortalized fibroblasts were then examined. Verapamil has been reported to be a potent inhibitor of P-glycoprotein and an inhibitor, but a less effective one, of MRP1 (36, 42, 43). MK-571, an antagonist of leucotriene D_4 receptor, has been shown to be a specific inhibitor of MRP1 (4). PGP-4008 [*N*-(1-benzyl-2, 3-dihydro-1H-pyrrolo [2,3-b]quinolin-4-yl)-2-phenyl-acetamide] (Alexis, San Diego, CA) is a newly developed, selective inhibitor of the P-glycoprotein (44). In WT fibroblasts, treatment with verapamil effectively enhanced the accumulation of calcein (Fig. 5*B*). MK-571 treatment also caused a significant but lesser increase in calcein accumulation. In contrast, treatment with PGP-4008 produced only a slight increase in calcein accumulation. In KO fibroblasts, inhibition of P-glycoprotein by verapamil or PGP-4008 resulted in a significantly increased accumulation of calcein, whereas exposure to MK-571 did not. In DKO fibroblasts, the accumulation of calcein was significantly enhanced only by inhibition of mrp1 by verapamil or MK-571. As expected, exposure of TKO fibroblasts to verapamil, MK-571, or PGP-4008 had no effect on calcein accumulation. Collectively, these results indicate that the predominant increase in the accumulation of calcein in fibroblasts is mainly because of the lack of mrp1 activity. The effects of P-glycoprotein deficiency on calcein accumulation appear to be minimal unless the function of mrp1 is also abrogated.

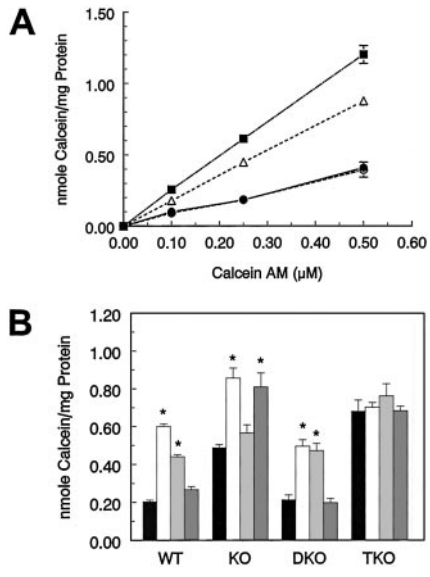

Fig. 5. *A*, steady-state cellular levels of calcein in WT (○), KO (△), DKO (●), and TKO (■) fibroblasts exposed to various extracellular concentrations of calcein AM for 1 h. *B*, the effects of MDR transporter inhibitors on the accumulation of calcein in immortalized fibroblasts. Cells were exposed to 0.25 μM calcein AM for 1 h in the presence or absence of 10 μM verapamil, 25 μM MK-571, or 10 μM PGP-4008. Data are the means ± SE of three to four independent experiments. *Black*, none; *white*, verapamil; *light gray*, MK-571; *dark gray*, PGP-4008. ∗, $P < 0.01$, significantly different from the value in the absence of inhibitors in each genotype.

Effects of MDR Inhibitors on the Sensitivity of WT and TKO Fibroblasts to Vincristine and Etoposide. To further characterize the contribution of mrp1 and P-glycoprotein to drug sensitivity, the effects of MDR inhibitors on the cytotoxic response of immortalized fibroblasts to vincristine and etoposide were examined. The sensitivity of WT and TKO fibroblasts to vincristine and etoposide in the presence or absence of MDR inhibitors is compared in Table 3. Treatment with verapamil or PGP-4008 alone at a concentration of 10 μM for 72 h did not result in appreciable toxicity to these

Table 3 The effects of verapamil (VPM) and PGP-4008 (PGP) on the sensitivity of WT and TKO fibroblasts to vincristine and etoposide

Agent	Inhibitor	WT		TKO		Relative sensitization[c]
		IC_{50} (nM)[a]	Fold sensitization[b]	IC_{50} (nM)	Fold sensitization	
Vincristine	None	17.0 ± 1.5		1.5 ± 0.06		
	PGP	2.5 ± 0.21	6.8	0.74 ± 0.02	2.0	3.4
	VPM	0.88 ± 0.06	19.3	0.67 ± 0.04	2.1	8.8
Etoposide	None	718 ± 19.2		132 ± 15.8		
	PGP	653 ± 62.3	1.1	177 ± 17.6	0.75	1.5
	VPM	233 ± 8.8	3.1	96 ± 17.6	1.4	2.2

[a] The IC_{50} of each agent was determined using the MTS assay. Values shown are means ± SE of three to six independent experiments.
[b] The fold sensitization was calculated as the ratio of the IC_{50} in the absence of inhibitors to the IC_{50} in the presence of inhibitors.
[c] The relative sensitization was calculated as the ratio of the fold sensitization of WT fibroblasts to the fold sensitization of TKO fibroblasts.

fibroblast cell lines. In the presence of verapamil, WT fibroblasts exhibited nearly complete sensitization to vincristine, with its IC_{50} (0.88 nM) approaching that of TKO cells (0.67 nM), whereas the sensitivity of TKO fibroblasts was considerably less affected. The effects of verapamil represented 19.3- and 2.1-fold increases in the sensitivity of WT and TKO fibroblasts, respectively, to vincristine. In contrast, verapamil caused a much lower sensitization of WT fibroblasts to etoposide (3.1-fold) and had a minimal effect on the sensitivity of TKO fibroblasts (1.4-fold). By comparison, PGP-4008 produced a moderate sensitization of WT fibroblasts to vincristine (6.8-fold), with no effect on the sensitivity of WT fibroblasts to etoposide (1.1-fold). In a manner analogous to verapamil, PGP-4008 exhibited a minimal effect on the sensitivity of TKO fibroblasts to either vincristine or etoposide. The marked sensitizing effects of verapamil on WT but not TKO fibroblasts suggest that the enhanced sensitivity of TKO cells to vincristine and etoposide is largely attributable to the lack of both mrp1 and P-glycoprotein activities in these cells.

Discussion

The establishment of immortalized embryonic fibroblasts lacking one or more of the MDR transporters provides a useful tool for evaluating the role of these transporters in the sensitivity of a single cell type to various anticancer agents without the complication of tissue-specific toxicity manifested by drug-treated knockout mice. These genetic knockout cell lines also contrast to most other cellular models in which the MDR transporters are overexpressed, thereby providing a different approach to examining the functional activity and interplay of these transporters.

Allen *et al.* (45) have recently reported the sensitivity of independently developed TKO fibroblasts to various compounds and anticancer agents. These authors developed the transporter-deficient fibroblast lines by 3T3-like procedures in which immortalized cell lines arose spontaneously by prolonged and repetitive cell passages. To minimize differences in growth rates incurred by spontaneous immortalization, we established immortalized transporter-deficient fibroblasts lines by transfection of early passaged primary cells with the SV40 genome. This methodology ensured that all fibroblast lines derived from primary cells consistently resulted from inactivation of the functions of p53 and Rb, rather than by random genetic alterations. This approach presumably minimized the development of clonal variations, which could potentially affect the assessment of drug sensitivities in these transporter-deficient cell lines. Although we observed that the immortalization process resulted in altered growth rates and increased drug sensitivity of all fibroblast cell lines from their primary embryonic fibroblast counterparts, the relative sensitivity among different genotypes of transporter deficiencies remained little changed from primary embryonic fibroblasts.

There are few examples of the ability of the MDR transporters to compensate for the absence of one another. Disruption of the *mdr1a* gene in mice has been reported to lead to an increased expression of the mdr1b P-glycoprotein in the kidney and liver but not in other tissues (46). In addition, an up-regulation of *MRP3* has been found to occur in liver when the function of MRP2 was deficient or impaired (16, 47). In our immortalized fibroblast lines, the expression level of P-glycoprotein in KO fibroblasts and the level of mrp1 in DKO fibroblasts were similar to those of WT fibroblasts (Fig. 2), suggesting that compensatory changes with respect to protein levels of these two transporters does not occur under normal physiological conditions. We also examined the possibility that other mrps were modified to compensate for the lack of mrp1 and P-glycoprotein in immortalized TKO fibroblasts. Considering mrps 1–7 as a group, mrp1, mrp2, mrp3, and mrp6 share the highest structural homology (48), and mrp1, mrp2, and mrp3 were shown to have overlapping specificities in the transport of certain anticancer drugs (19–23, 37). RT-PCR analyses demonstrated that a similar profile of the expression of mrps 2–7 occurred between WT and TKO fibroblasts, with the expression levels of mrp2, mrp3, and mrp6 being very low in both fibroblast cell lines (Fig. 3, *A* and *C*). Therefore, it is unlikely that mrp2, mrp3, and mrp6 in immortalized fibroblasts play a major role in drug sensitivity. An apparent slight elevation of mrp3 in TKO fibroblasts may represent a change that at least compensates, in part, for the loss of mrp1 and the P-glycoprotein in these cells. However, the significance of such a slight change remains to be determined because in the presence of cytotoxic selection, a compensatory increase in the expression of one of the other transporters may possibly occur. Adult mouse tissues exhibited expression patterns of Mrp proteins distinct from immortalized fibroblasts. Mrp2, mrp3, and mrp6 were the predominant mrps present in liver, whereas mrp3, but not mrp2 and mrp6, were expressed in lung (Fig. 3*B*). These

observations are consistent with reports on the tissue-specific expression of the mrps by others (16–18, 49).

Our data on drug sensitivity and calcein accumulation strongly support the notion that mrp1 and P-glycoprotein are compensatory transporters for vincristine, etoposide, and other common substrates. Thus, fibroblasts with disruptions of both mrp1 and P-glycoprotein exhibit hypersensitivity to vincristine and etoposide and a substantial increase in calcein accumulation. By comparison, fibroblasts solely lacking either mrp1 or P-glycoprotein are relatively less sensitive to these drugs and display a lesser increase in calcein accumulation (Figs. 4 and 5*A*). Given that deficiency in mrp1 in fibroblasts does not cause overt compensatory change in the expression of P-glycoprotein and *vice versa*, our results suggest that functional substitution of one transporter for the absence of the other does occur in the presence of common substrates. Thus, genetic or functional abrogation of both mrp1 and P-glycoprotein should incur synergistic consequences when challenged with cytotoxic agents that are substrates for both transporters.

We observed that DKO fibroblasts were slightly more sensitive (2.5-fold) to vincristine than KO fibroblasts (1.7-fold). Inversely, KO fibroblasts were more sensitive (3.4-fold) to etoposide than DKO fibroblasts (1.3-fold). These results imply that in the presence of both transporters, vincristine is transported more effectively by P-glycoprotein, whereas etoposide is transported preferentially by mrp1. This speculation is additionally supported by studies using calcein AM and its hydrolysis product calcein as substrates for mrp1 and P-glycoprotein. The study of Feller *et al.* (50) indicates that mrp1 can actively efflux highly negatively charged calcein from the cytoplasm, in addition to its ability to efflux neutral lipophilic calcein AM, whereas P-glycoprotein can only transport calcein AM (40). A major increase in the calcein level observed in KO fibroblasts demonstrates that the transport of calcein and calcein AM is mainly mediated by mrp1. In contrast, no increase in the level of calcein occurred in DKO fibroblasts compared with WT fibroblasts at either 5, 15, or 60 min of incubation (time course of calcein accumulation not shown). The lack of both mrp1 and P-glycoprotein in TKO fibroblasts resulted in an accumulation of calcein that was additionally increased over the calcein level in KO fibroblasts (Fig. 5*A*). Our results using established MDR inhibitors also demonstrated that P-glycoprotein-specific inhibitor PGP-4008 caused only a slight increase in calcein accumulation in WT fibroblasts but a significant increase in calcein accumulation in KO fibroblasts (Fig. 5*B*). These findings suggest that P-glycoprotein is subordinate to mrp1 in the efflux of calcein/calcein AM Therefore, in DKO cells mrp1 activity can mask or compensate for the absence of P-glycoprotein in effluxing calcein AM.

The marked effects of verapamil in sensitizing WT but not TKO fibroblasts to vincristine and etoposide further support the concept that the enhanced sensitivity of TKO fibroblasts to these antineoplastic agents is attributable to deficiencies in both mrp1 and P-glycoprotein. Similar effects of verapamil on calcein accumulation in fibroblasts were also observed (Fig. 5*B*). In drug sensitivity assays, we found that verapamil completely sensitized WT fibroblasts to vincristine but only partially sensitized WT fibroblasts to etoposide. The incomplete sensitization of WT fibroblasts to etoposide by verapamil may be because verapamil is less effective as an inhibitor of mrp1 (36, 42, 43). In support of this possibility, PGP-4008, a P-glycoprotein-specific inhibitor, caused a substantial sensitization of WT fibroblasts to vincristine but had no effect on the sensitivity of WT fibroblasts to etoposide (Table 3). These findings collectively suggest that mrp1 may preferentially contribute to etoposide transport. Therefore, after treatment with verapamil, the remaining mrp1 activity in WT fibroblasts may be sufficient to prevent complete sensitization to etoposide.

The findings emphasize that mrp1 and the P-glycoprotein can functionally compensate for each other in the face of pharmacological challenge. Therefore, these transporters can protect cells and tissues from the effects of lethal concentrations of toxins and thereby play a major role in determining the sensitivity of cells to cytotoxic agents. However, both mrp1 and P-glycoprotein may be functionally substituted by other ABC transporters with respect to endogenous substrates and therefore dispensable under normal physiological conditions. This notion is strongly supported by findings with knockout mice. Thus, mice with disruptions of both mrp1 and P-glycoprotein develop normally and display no abnormalities (30, 51). Additional characterization of other mrps in various tissues of triple knockout mice should provide more insight into the physiological and pathological functions of the MDR superfamily.

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