Expression of Functionally P-Glycoprotein in MA104 Kidney Cells

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Rhesus monkey kidney MA104 cells are a polarized epithelium with some unusual characteristics, including a resistance to ouabain, although their Na+-K+-ATPase has normal affinity with this drug. This work suggests that MA104 cells have high expression of functionally P-glycoprotein in their membranes. This was established using four complementary methods to investigate the expression and function of P-glycoprotein in these cells. MA104 cells were strongly resistant to vincristine, which could be reversed by three known P-glycoprotein modulators: verapamil, cyclosporin A and trifluoperazine. In addition, MA104 cells accumulate little rhodamine 123, and the incubation with verapamil increased this accumulation. The mdr1-mRNA was detected by reverse transcription-polymerase chain reaction and a subcloned 283-bp product was identified. Its nucleotide sequence was compared with the related region of human mdr1, showing a high identity (96%) between the two sequences. The expression of P-glycoprotein in the cell membrane was observed by Western blot and immunofluorescence. The results taken together suggest that MA104 cells intrinsically have a high expression of functionally P-glycoprotein in their membranes.

Introduction

MA104 is a cell line derived from the kidney of rhesus monkey embryo. It expresses several characteristics of polarized epithelial cells, such as the formation of "domes", the establishment of a transmonolayer electrical resistance and polarized maturation of enveloped virus (Roth et al., 1987). This cell line has been extensively used to study virus replication and is being currently employed to study the membrane-associated folate binding protein (WesteRhof et al., 1995; Smart et al., 1996a) as well as the role of caveolin, a protein involved in the transport of newly synthesized cholesterol (Smart et al., 1996b). Furthermore, MA104 cells exhibit an unusual feature: they are resistant to ouabain, although their Na+-K+-ATPase have normal affinity with this drug (Contreras et al., 1995a), being also able to protect ouabain-sensitive Madin-Darby Canine Kidney (MDCK) cells in co-culture.

In an attempt to characterize these cells further, it was observed by us (unpublished data) that they were also resistant to a number of drugs that inhibit ion transport, such as furosemide and thapsigargin. It is known that thapsigargin is a substrate to P-glycoprotein (Pgp), a transmembrane protein capable of actively transporting chemotherapeutic and other unrelated drugs outside of the cells, leading to a multidrug resistance (MDR) phenotype, the major cause of chemotherapy failure in many tumors (Chan et al., 1994; Gutheil et al., 1994; Neyfakh, 1988). Pgp is also highly expressed in some normal tissues, such as human adrenal cortex and the luminal membranes from bile canals.

Abbreviations: Pgp, P-glycoprotein; VCR, Vincristine; VP, verapamil; CSÃ, cyclosporin-A; TFP, trifluoperazine; Rho 123, rhodamine 123; FCM, flow cytometry analysis; RT, reverse transcription; PCR, polymerase chain reaction; RPA, ribonuclease protection assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MDCk, Madin-Darby Canine Kidney; EDTA, ethylenediaminetetraacetic acid; SCH 28080, 3-(cyanomethyl)-2-methyl-6-(phenylm ethoxy)-imidazo[1,2-a] pyridine; DTT, 1,4-dithio-threitol; HEPES, N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid; Tris, trishydroxy-methyl-aminomethane.

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naliciuli, proximal renal tubular and colonic epithelia. There is also a low expression of Pgp in some distal tubule and collecting duct cells (Thiebaut et al., 1987; Cordon-Cardo et al., 1990). Its physiologic function in renal cells is not entirely clear, but the function of Pgp at these sites appears to be related to the secretion of hormones and protection against toxins, as the expression is restricted to the luminal pole. This paper presents evidence that MA104 cells have a high expression of Pgp in their membranes.

Materials and Methods

Cells and culture conditions

MA104 cells were a gift of Dr. M. Cereijido (Center for Research and Advanced Studies, Mexico). Cells (1x10^5 cell/ml) were grown in Dulbecco's Modified Eagle Medium-DMEM (GIBCO, Grand Island, NY, USA) with 20 mM N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES – GIBCO, Grand Island, NY, USA) and 10% fetal bovine serum (GIBCO, Grand Island, NY, USA), in disposable plastic bottles at 37 °C for 4 days. For each experiment, cells were harvested using trypsin-EDTA (ethylenediaminetetraacetic acid) (GIBCO, Grand Island, NY, USA) and plated on 24-well chambers, at a concentration of 2x10^5 cells/ml (1 ml/well). To assess resistance to vincristine, the experiments were performed after 24 h of incubation in these chambers.

K562, a human erythroleukemia cell line, and K562-Lucena 1, derived from K562 and overexpressing P-glycoprotein (Maia et al., 1996), were used as negative and positive controls, respectively, in Western blotting assays. These cells were grown in suspension in the same conditions described for MA104 cells.

Resistance to vincristine

Vincristine (VCR, vincristine sulfate, Sigma, St. Louis, USA) was added in various concentrations (100–500 nm) to 24 well-chambers containing 2x10^5 MA104 cells per well. In some experiments, the Pgp modulators verapamil (VP, Verapamil hydrochloride, Sigma, St. Louis, USA), cyclosporin-A (CSA, Sandimmun®, Sandoz, Switzerland) or trifluoperazine (TFP, trifluoperazine hydrochloride, Sigma, St. Louis, USA) were also added. After 48–72 h of incubation, the cells were harvested using trypsin-EDTA and the cell number and viability was measured by trypan blue exclusion. The data were analyzed by two-way analysis of variance (ANOVA), considering the treatments as factors. The significance of the differences was verified by the Bonferroni t-test (Zar, 1996).

Rhodamine 123 accumulation

The accumulation of rhodamine 123 (Rho 123; 2-[6-amino-3-imino-3H-xanthen-9-yl] benzoic acid methyl ester) was measured by flow cytometry. The cells were plated at a concentration of 5x10^5 cells/ml in 25 mm Petri dishes and incubated for 24 hours for cell anchorage. The medium was then replaced by a fresh medium with 200 ng/ml Rho 123 and the cells incubated for 1h in the presence or absence of the Pgp modulator verapamil (10 μM). After incubation, the cultures were washed twice in phosphate buffered saline (PBS) and left to extrude the dye in dye-free medium for 30 min at 37 °C. After incubation in dye-free medium, the cells were harvested using trypsin-EDTA, washed and suspended in PBS. Flow cytometry analysis (FCM) was performed with an Epics Elite cytometer (Coulter Corp., FL, USA) equipped with an air-cooled Argon Laser tuned to emit 15 mW at 488 nm. The fluorescence was measured through a 600 nm long pass filter. The analysis was performed by the software Multicycle (Phoenix Flow Systems, CA, USA).

Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA from MA104 cells was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). RNase-free DNAse I (1 Unit/μl) was used to treat the isolated RNA for 1 hour to eliminate contamination with genomic DNA. cDNA was synthesized from 1 μg of total RNA using Superscript Reverse Transcriptase (GIBCO, NY, USA) at 37 °C for 60 min, and the reaction was terminated by heat at 95 °C for 10 min. The cDNA synthesis was primed with oligo-dT. The primers used for PCR amplification were designed based on the published nucleotide sequence of human mdr1 (Clen et al., 1990). The sense primer (5’-CACACCTGGGGCATCGTGT-3’) is located in
the exon 26 and the antisense primer (5'-GCTGACGTGGCTTCATCC-3') is located in the exon 27, corresponding to nucleotides 3332 to 3344 and to 3597 to 3614, respectively, of human mdr1 sequence. PCR was performed with 40 cycles of denaturation (94 °C, 1 min), annealing (51 °C, 1 min) and extension (72 °C, 1 min). The PCR product was ligated into PCRScrip (Stratagene, CA, USA) according to the manufacturer’s instructions and sequenced using the Sequenase 2.0 kit (Amer sham, IL, USA).

**Southern Blotting**

Southern blotting was performed on PCR-amplified products analyzed and separated by electrophoresis on 1.0% (wt:vol) agarose gels. DNA was transferred to Hybond N+ (Amersham, IL, USA). The blots were prehybridized in QuikHyb (Stratagene, CA, USA) for 10 min at 37 °C. The internal oligonucleotide (5'-CAGGAGCCCATCCTG-3') from the internal sequence of MA104 mdr1 (MDR-MA) PCR product was used as probe. The oligonucleotide was end labeled with [γ-32P] ATP (111 TBq/mmol, DuPont-NEN, MA, USA) with T4 polynucleotide kinase. The probe was added to the QuikHyb along with 1 jig of salmon sperm DNA. MDR-MA was hybridized for 1 hour at 50 °C. The blots were washed two times for 15 min in 2xSSC and 0.1% SDS at room temperature. The blot was exposed to autoradiography film at -80 °C.

**Ribonuclease protection assay (RPA)**

The fragment of 283-bp corresponding to mdr1 of MA104 cells (related to nucleotides 3332 to 3614 of human mdr1 gene) was ligated to PCRScrip SK(+) (Stratagene, IL, USA) and used to transform Epicurian Coli XL1-blue supercompetent cells (Stratagene, IL, USA). After checking the nucleotide sequence with Sequenase version 2.0 (US Biochemicals, Ohio, USA), the fragment served as template for antisense RNA synthesis. Probes were prepared by linearization of 1 μg of template DNA by restriction enzyme digestion with Not I (GIBCO BRL, NY, USA). Following the Maxscript kit protocol (Ambion, Texas, USA), all templates were transcribed in vitro in the presence of [α-32P]UTP (29.6 TBq/mmol, Amersham, IL, USA) with T7 RNA polymerase generating radiolabeled antisense RNA probe. The probe was evaluated with 10 μg of yeast tRNA in the presence and absence of RNase A and T1 following the RPA II™ kit protocol (Ambion, Texas, USA). Aliquots of 30 μg of total RNA from MA104 cells were mixed with radiolabeled antisense probe (1x10^5 cpm/sample) and hybridized at 45 °C for 18 hours. A combination of RNase A and T1 (RPA II kit, Ambion, Texas, USA) was used to treat the samples at 37 °C for 30 min. Analysis of the protected fragment was made in 8M urea/5% polyacrylamide gels. The gels were transferred to chromatography paper and exposed to X-ray films with an intensifying screen at -80 °C. The undigested MDR-MA probe was 96-bp larger than the digested probe.

**Western blot analysis**

Expression of Pgp in MA104, K562 and Lucena 1 cells was assessed by immunoblotting using the mouse anti-Pgp monoclonal antibody JSB-1 (SIGNET Laboratories, MA, USA), which recognize an internal epitope of Pgp. After aspiration of the culture media, confluent cells were washed 3 times with PBS (pH 7.4) at room temperature, scraped, and centrifuged at 8,000×g for 90s. The cell pellets were flash frozen in liquid nitrogen and kept at -80 °C until use. Two cell lines were used as positive and negative controls: K562, a human erythroleukemia cell line that does not express Pgp and K562-Lucena 1 cells, derived from K562 and overexpressing Pgp (Maia et al., 1996). In this case, suspension growing cells were washed three times with PBS by centrifugation, and the cell pellets frozen and kept at -80 °C until use. Whole cell extracts were prepared from frozen cell pellets solubilized in ice cold 250 mM sucrose, 1 mM EDTA and 20 mM imidazole, pH 7.2, containing the protease inhibitors (Sigma, St. Louis, USA): 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mM benzamide, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1μg/ml aprotinin, and 1μg/ml chymostatin, pH 7.2, and then homogenization was carried out in the cold using Potter apparatus. The homogenate was centrifuged at 1,000×g for 10 min. The supernatant was saved, the pellet was suspended in 3 volumes of the same medium, and the centrifugation was repeated. The supernatants were mixed and centrifuged at 10,000×g for 20 min to
separate mitochondria. The ensuing supernatants were centrifuged at 100,000×g for 1 hour, the pellets containing the cell membranes were suspended in the ice-cold homogenization buffer, and protein concentration was determined with bicinchoninic acid protein assay (Pierce Chemical) using bovine serum albumin (BSA) as standard. All the extracts were solubilized by heating at 95 °C for 2 min in sample buffer: 1.5% sodium dodecyl sulfate (SDS), 10 mM Tris(hydroxymethyl)aminomethane (Tris-Cl) pH 6.8, 0.6% dithio-L-threitol (DTT) and 6% glycerol. The membrane proteins (25 µg/lane) were subjected to 7% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 9% nonfat milk in PBS and incubated with the human MDR1-specific monoclonal antibody JSB-1 (SIGNET Laboratories, MA, USA) 1:1,500 in PBS and 0.05% Tween 20, pH 7.4, at 4 °C for 12–24 h. The secondary antibody was a horseradish peroxidase-sheep anti-mouse IgG (1:2,000 dilution for 1 h (Amersham, IL, USA). Detection was by enhanced chemiluminescence (ECL, Amersham, IL, USA). Each immunoblot was at least performed in triplicate. The specific bands were not detected in the experiments made in absence of antibody discarding unspecific binds.

**Immunofluorescence**

Cells grown on coverslips were fixed in paraformaldehyde/sucrose 4% and incubated with triton X-100 0.2% to permeabilize cell membrane (since the JSB-1 antibody recognizes an internal epitope of Pgp, it is necessary to permeabilize the cell membrane in order to expose this epitope). After washing, the cells were incubated with bovine serum albumine BSA (GIBCO, Grand Island, NY, USA) 5% in PBS for 30 minutes, washed and incubated overnight at 4 °C with the human JSB-1 antibody (5 µg/ml). Goat anti-mouse FITC-conjugated IgG (SIGMA, St. Louis, USA), was used for detection. The fluorescence was visualized under a confocal laser scanning microscope (Zeiss, CLSM310).

![Fig. 1. Resistance of MA104 cells to vincristine (VCR). Main figure: 2×10⁵ cells/ml were incubated with various concentrations of VCR in 24 multiwell plates (1 ml/well) for 72 hours. After this time, cells were harvested with trypsin and the cell number was counted. Insert: MA104 cells were incubated for 48 h with 500 nM VCR in the presence of Pgp inhibitors. VP – verapamil; CSA – cyclosporin A; TFP – trifluoperazine. * Statistically significant when compared to control (p<0.05). ** Mean values not statistically different. Data obtained from 3 different experiments.](image-url)
Results

Assessment of the MDR phenotype

The multidrug resistance phenotype of MA104 cells was evaluated by testing its resistance to vincristine, a classical substrate for Pgp. The data shown in Fig. 1 indicate that MA104 cells are resistant to VCR concentrations up to 500 nM and that this resistance is reverted by three different Pgp modulators: TFP (10 μM), CSA (20 nM) and VP (10 μM), with VP being the best.

Accumulation of Rho 123

To examine the activity of Pgp in MA104 cells the functional assay of extrusion of Rho 123 was performed. Fig. 2 (upper panel) shows that MA104 cells accumulate very little Rho 123 (Fig. 2b). Only when they were incubated in the presence of VP there was some accumulation of the dye (Fig. 2c).

Detection of mdr1 in MA104 cells by RT-PCR and RPA

To detect the presence of mdr1 in MA104 cells, RNA isolated from these cells was amplified by RT-PCR using primers located in exons 26 and 27 of human mdr1 (Clen et al., 1990), spanning the second nucleotide binding domain (NBD-2). The amino acid sequence of that region is usually conserved between different species. The expected 283-bp fragment was observed by RT-PCR and confirmed by southern blotting using radiolabelled oligonucleotide to the internal sequence of that fragment (Fig. 3A). A 283-bp PCR product was subcloned and its nucleotide sequence was identified by sequencing. The comparison between the amplified region of MDR-MA and related region of human mdr1 (Fig. 3B) illustrates the high degree of identity between these two sequences (96%), the differences consisting of nine mismatches. A change of G, C, C, A, C, T, T, A and T for A, G, T, C, T, C, G and C, respectively, in the positions 3382, 3383, 3435, 3447, 3459, 3465, 3501, 3503 and 3561 of human MDR-1 sequence was seen. The alterations in the nucleotides 3382 and 3383 lead to a change from alanine in the human sequence to serine in the MA104 sequence, and the alteration of nucleotide 3503 leads to a change from lysine to arginine. The other nucleotide differences do not modify the amino acid sequence of the amplified fragment.

To prove the existence of MDR-MA in RNA levels the RNase protection assay (RPA), a very sensitive and specific method to detect mRNA expression, was performed. If some mistakes in the nucleotide sequence of MDR-MA sequence were introduced by the taq polymerase during the PCR reaction, we should not be able to detect the expected fragment by ribonuclease protection assay because of the RNase digestion of single strand RNA. The expression of MDR-MA was detected hybridizing 30 μg of total RNA from MA104 cells with 32P-labeled antisense RNA probe (Fig. 4).

The mere existence of MDR1-mRNA does not mean that Pgp is expressed at the cell membrane or is in a functional state (Mickley et al., 1989; Marks et al., 1995; Klimecki et al., 1994). There-
Fig. 3. a. Southern blotting of amplified MDR-MA (283-bp) reverse transcription-polymerase chain reaction (RT-PCR) product in MA104 cells. RT(-): Control, where reverse transcriptase step was eliminated. b. comparison between human MDR-1 and MDR-MA sequences corresponding to nucleotides 3332 to 3614 of human MDR-1 cDNA.

Expression of Pgp in the cellular membrane

The expression of Pgp was demonstrated by immunoblotting (Fig. 5a). For comparison, the same assay was performed in other two related cell lines, K562, an erythroleukemia cell line that do not express MDR phenotype and its derived multidrug resistant K562-Lucena 1 cell line. As expected, Pgp is abundantly expressed in MA104 and K562-Lucena 1 cells. To determine the expression of Pgp in the cell membrane an immunofluorescence assay was performed (Fig. 5b). It was observed that Pgp is expressed not only in the cell membrane of MA104 cells, but also in an internal region. According to Kim et al. (1997), Pgp usually undergoes constitutive endocytosis and under steady state about 30% of cellular Pgp is intracellular.

Discussion

A number of methods have been described to detect Pgp, the advantages and disadvantages of each one were analyzed by Chan et al. (1994). In the present study, five methods were applied to detect the expression and function of Pgp in the membrane of MA104 cells: 1) the use of Pgp mod-
Fig. 4. MA104 cells mRNA detection by ribonuclease protection assay performed with 30 μg of total RNA. A 283-bp RT-PCR fragment was used as template for in vitro transcription of labeled antisense RNA probe to detect MDR-MA. Detection of 283-bp band shows that MDR is present in MA104 cells. Lane 1 corresponds to RNA marker. Lanes 2 and 3 demonstrate the evaluation of the probe with 10 μg of yeast tRNA in the absence and presence of RNase A and T1, respectively. The undigested probe was 96-bp larger than the protected fragment. Lane 4 shows MDR-MA expression in MA104 cells total RNA.

The relation (if any) between the resistance of MA104 to ouabain and our finding that these cells are multidrug resistant (MDR) remains to be proved, but we observed that the resistance of MA104 cells to ouabain could not be reversed by Pgp inhibitors (data not shown). Brismar et al. (1995) showed that a multidrug resistant K562 cell line obtained by them had a ouabain-resistant uptake of the potassium analogue 203H which could be reversed by 3-[aminosulfonyl]-5-[butylamino]-4-phenoxybenzoic acid (bumetanide), suggesting the presence of Na+-K+-2Cl- cotransport in those cells. However, the authors also could not demonstrate a direct relation between the resistance to ouabain and the MDR phenotype. In another study (Abrahamse et al., 1995) it was suggested the existence of a K+-ATPase insensitive to either ouabain (inhibitor of Na+-K+-ATPase) or 3-(cyclohexylmethyl)-2-methyl-8-(phenylmethoxy)-imidazo-[1,2,a]pyridine (SCH 28080, inhibitor of H+-K+-ATPase) in colonic epithelial cells, which are known to have Pgp constitutively.
It is well known that some transport inhibitors used in vesicles or broken cell preparations lack their effectiveness when used in intact cells. We observed that MA104 cells were also resistant to other transport inhibitors, such as furosemide (5-[aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)-amino]-benzoic acid), which inhibits the Na+-K+-2Cl⁻ cotransport, amiloride hydrochloride, which blocks sodium channels at the luminal membrane and thapsigargin, which inhibits the Ca²⁺ ATPase from sarco/endoplasmatic reticulum (data not shown). These results point to a possible role of Pgp or other Pgp-like proteins in this apparent contradiction. Thus, when studying the effects of ion transport modulators or other possible Pgp substrates in whole cells, it is desirable to investigate the presence of a MDR phenotype.

Although the role of Pgp in tumor MDR phenotype has been extensively studied, its role in normal cells is not yet understood. It is believed that Pgp may function in the extrusion of xenobiotics or toxins, but a number of studies have demonstrated other singular characteristics of Pgp-expressing cells, such as:

I. alterations in plasma membrane electrochemical potentials of Pgp expressing cells (Roepe, 1995);
II. hyperosmotic shock and acidic shock increase the expression of Pgp in the cell membrane (Wei and Roepe, 1994);
III. Pgp seems to function as an ATP channel (Abraham et al., 1993);
IV. some authors suggest that it is related to a volume-regulated chloride channel (Valverde and Sepulveda, 1992; Han et al., 1996);
V. Pgp-expressing cells have altered Ca²⁺ metabolism (Koch et al., 1986), altered internal pH (Wei and Roepe, 1994) and ouabain-insensitive K⁺ transport (Brismar et al., 1995; Abrahamse et al., 1995), although none of those authors could directly correlate these findings with the expression of Pgp.

Despite the importance the above findings may have to the understanding of the physiological role of naturally occurring Pgp, the majority of the studies were done with tumor cells transfected with mdr1 gene or made resistant by successive incubations with increasing concentrations of drugs such as vincristine or doxorubicin, which lead to the overexpression of other proteins. Thus, we believe MA104 cells may be a powerful model to study the physiological role of Pgp in renal cells.

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