Molecular Evidence and Functional Expression of P-Glycoprotein (MDR1) in Human and Rabbit Cornea and Corneal Epithelial Cell Lines

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PURPOSE. Efflux pumps such as P-glycoprotein (P-gp; MDR1) are believed to be a major barrier to drug delivery. The purpose of this work was to determine whether cornea and corneal epithelial cells express the functionally active P-gp efflux pump.

METHOD. Cultured rabbit primary corneal epithelial cells (rPCECs) and a corneal cell line (Statens Seruminstitut rabbit cornea [SIRC] cells) were selected as the model. Rhodamine-123 (Rho-123), a P-gp substrate, was used as a P-gp probe. To confirm gene expression, RT-PCR was performed with appropriate pairs of primers for rabbit and human MDR1. Subcloning, sequencing, and protein sequence determination were performed to confirm Pgp.

RESULTS. Permeability of [3H] cyclosporin A (CsA) across SIRC cells was found at 1.74 × 10⁻¹⁶ cm/s in the apical-to-basolateral and 5.1 × 10⁻¹⁶ cm/s in the basolateral-to-apical directions. Uptake of Rho-123 across both SIRC cells and rPCECs was time and temperature dependent. Rho-123 uptake in SIRC cells was 14.4 picomoles/mg protein and in the presence of CsA (10 μM) was 70.8 picomoles/mg protein at 3 hours. Uptake in rPCECs was the highest at 3 hours. Western blot analysis indicated a 170-kDa band confirming the presence of P-gp. Human cornea was also checked for the presence of P-gp. RT-PCR data indicated one single band, which was subcloned and sequenced to confirm the presence of P-gp. The protein sequence deduced from the fragment product indicated more than 89% homology with human MDR1.

CONCLUSIONS. Functional and molecular characterization showed the existence of P-gp in human cornea, rabbit cornea, and a rabbit corneal cell line. This knowledge of the existence of P-gp will help in development of better ocular drug delivery strategies.

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expression on human cornea and functional characterization of this protein on rabbit and human corneas have not been reported.

In this study, we present the molecular evidence and functional expression of P-gp on rabbit cornea and rabbit corneal epithelial cells (both primary culture and established cell line). In addition, we found for the first time that human corneal epithelium also expresses Pgp. In this study, rhodamine-123 (Rho-123; a fluorescent P-gp substrate) was selected to examine the function of Pgp in vitro. Rho-123 has been used widely as a P-gp substrate, because it does not bind to the efflux pump (hence does not act as an inhibitor) and can be detected in very low amounts. All the in vitro studies were performed in primary cultured rabbit corneal epithelial cells (rPCECs). We have also compared these data with those obtained from a commercially available rabbit corneal epithelial cell line (Statens Seruminstitutt Rabbit Cornea; SIRC). It is a well-known and established cell culture model that mimics corneal epithelium. This cell line has been used for in vitro studies to assess corneal physiology, transport, immunology, cytotoxicity, and pharmacology.

### MATERIALS AND METHODS

#### Animals

New Zealand white male rabbits weighing between 2 and 2.5 kg were obtained from Myrtle’s Rabbitry (Thompson Station, TN). Human corneas were obtained from Midland Lion’s Eye Bank (Kansas City, MO). All studies involving rabbits were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. CsA, quinidine, verapamil, chlorpromazine, testosterone, progesterone, probenecid, indomethacin, triethylammonium chloride (TEA), 1-methyl-4-phenyl pyridinium iodide (MPP+), sodium azide, 3-O-methyl-D-glucopyranoside (3-MG), and Rho-123 were purchased from Sigma-Aldrich (St. Louis, MO). 1H-mannitol (specific activity: 50 mCi/mmol) and [3H]-methylpyridine (specific activity: 8 Ci/mmol) were purchased from Amersham (Piscataway, NJ). Stock solutions of Rho-123 were prepared in Dulbecco’s modified phosphate-buffered saline (DPBS). CsA (2 mM) was first dissolved in ethanol (Fisher Scientific, Fair Lawn, NJ), and then aliquots were diluted in DPBS to a final concentration of either 5 or 10 μM for inhibition studies.

SIRC cells (passage 410) were obtained from American Type Culture Collection (CCL-60; ATCC, Rockville, MD). The growth medium, minimum essential medium (MEM), was obtained from Life Technologies (Grand Island, NY). Penicillin, streptomycin, sodium bicarbonate, lactalbumin, amphotericin B, polymyxin B sulfate, and HEPES were obtained from Life Technologies. 12-well culture plates (1.1 cm2 growth area), and polyester membranes (Transwell; pore size, 0.4 μm; diameter, 6.5 mm), 24-well culture plates (1.1 cm2 growth area), and polyester membranes (pristane, 0.4 μm; diameter, 1 cm) were obtained from Corning Costar (Bedford, MA). The vector (pGEM-T-Easy) and EcoRI were obtained from Promega (Madison, WI).

#### Cell Culture

**Conventional Culture of SIRC Cells.** SIRC cells were grown at 37°C in a humidified atmosphere of 5% CO2 and 90% relative humidity in MEM containing 10% (vol/vol) non–heat-inactivated FBS, lactalbumin enzyme hydrolysate, penicillin (100 U/mL), and streptomycin (100 μg/mL). Confluency was achieved in 5 days, and semiconfluent cells were subcultured (subculturing ratio, 1:5) every 4 days with 0.25% trypsin containing 0.55 mM EDTA. Cells were seeded at a density of 2.5 × 105 cells/well in 12-well culture plates and were also plated on clear polyester membranes (pore size, 0.4 μm) placed in culture dishes that were coated with rat tail collagen type I followed by human fibronectin before subculturing. Cells were allowed to grow for 10 days, to differentiate completely before either uptake or transport experiments were performed. Cells of passages 41 to 430 were used throughout the study.

**Primary Culture of Rabbit Corneal Epithelial Cells.** Corneas from New Zealand White (NZW) rabbits were excised and washed thoroughly with DPBS, blotted dry and transferred to sterile culture dishes containing 0.5 mL trypsin (0.25%) or 1.2 U/mL protease (Dispase II, Roche Molecular Biochemical). The corneas were placed upside down (with the concave surface touching the protease solution) and incubated at 37°C for 30 minutes. The epithelial cells were stripped off with gentle scraping from peripheral areas (1–1.5 mm from the limbus) to the center. Care was taken to peel only the epithelial layer and not the underlying stromal layer, because that would contaminate the epithelial cells with keratinocytes. Cells were washed with MEM and placed in culture dishes. After 12 hours (when most of the cells had detached to the bottom), MEM was removed and fresh MEM supplemented with insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL), amphotericin B (0.25 μg/mL), polymyxin B sulfate (0.5 μg/mL), penicillin (100U/mL), streptomycin (100 μg/mL), human recombinant epidermal growth factor (10 ng/mL), and bovine pituitary extract (50 μg/mL) were added. The media was changed twice a week, and the cells were subcultured every 7 to 10 days (subculture ratio, 1:5). Cells reached senescence by passages 7 to 10, and therefore passages 2 to 6 were used for all further experiments.

**Immunoprecipitation Western Blot**

Confluent SIRC cells and rPCECs grown on T-75 (75 cm² growth area) were washed twice with PBS and harvested with a cell scraper into 5 mL of PBS. The cell suspension was centrifuged at 1500 rpm for 10 minutes, and the pellet was resuspended in freshly prepared lysis-immunoprecipitation buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 2 mM Na2VO4, 5 mM NaF, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, and 1:1000 protease inhibitor cocktail) for 15 minutes on ice. The cells were ultrasonicated (Branson Cleaning Equipment Co., Shelton, CT) for 30 seconds and then centrifuged at 12,000 rpm for 10 minutes at 4°C. The membrane fraction and supernatant were collected and stored at −80°C until used. Protein content was determined using Bradford method (Bio-Rad protein estimation kit; Bio-Rad, Hercules, CA). Twenty micrograms of protein (150 μL lysis) with 150 μL immunoprecipitation buffer was immunoprecipitated with a monoclonal antibody to human anti-Pgp (RDI-PRO57043; Research Diagnostics, Inc., Flanders, NJ) overnight at 4°C. This monoclonal antibody (clone determination: JSB-1) reacts with a conserved cytoplasmic epitope of the plasma membrane–associated Pgp. The tubes were then incubated with an immunoadsorbent (protein A-Sepharose 6MB; Sigma-Aldrich, St. Louis, MO) for 2 hours at 4°C, and Sepharose beads containing antigen–antibody complexes were collected by centrifugation, washed three times with immunoprecipitation buffer, resuspended in 30 μL of SDS sample buffer (62 mM Tris [pH 6.6], bromophenol blue), and boiled for 5 minutes. All the immunoprecipitated samples and molecular weight protein markers were separated by SDS polyacrylamide gel electrophoresis (7.5% Tris-glycine gels for 2 hours at 100 V). Protein was transferred onto nitrocellulose membranes for 4 hours at 30 V on ice. Protein transfer efficiency was checked by staining the nitrocellulose membranes in 0.2% ponceau S (in 5% wt/vol trichloroacetic acid and 5% wt/vol sulfosalicylic acid) for 10 minutes. The portion of the blot containing Pgp samples was incubated in freshly prepared blocking buffer (5% wt/vol BSA and 5% nonfat dry milk in Tris-buffered saline) for 1 hour at room temperature.

The blot was then probed with anti-Pgp antibody overnight at 4°C. After five 10-minute washes in TBST (Tris-buffered saline + 0.1% Tween 20), the membranes were probed with secondary antibody in TBST (1:2000 anti-mouse IgG–horseradish peroxidase [HRP]). The blots were finally washed three times (15 minutes each) with TBST. A Western blot amplification kit (Bio-Rad) was used to develop the blots, according to the manufacturer’s protocol.
Uptake Experiments

Uptake studies were conducted based on standard protocols, with modifications. Briefly, 12 to 14 days after seeding, the medium was aspirated and the cells were washed with DPBS at 37°C. Rho-123 solution was prepared at a concentration of 50 μM in DPBS. Cells were then equilibrated in 1 mL of DPBS for 30 minutes at 37°C for control experiments or equilibrated with 1 mL solution of appropriate inhibitor (10 μM CsA, 100–200 μM testosterone, 1–1000 μM MPP⁺, 1–1000 μM TEA) in DPBS. The time-dependent accumulation of Rho-123 was determined with and without the inhibitor. At the end of the experiments, the drug solution was removed, and the cells were washed three times with 2 mL of ice-cold stop solution (210 mM KCl, 2 mM HEPES [pH 7.4]) to stop the cellular uptake. Cells were then solubilized in 1 mL of lysis solution (0.3 M NaOH, 0.1% Triton X-100). The lysate was transferred to a 96-well plate and was assayed using a 96-well fluorescent microplate reader. Rho-123 fluorescence was measured at excitation-emission wavelengths of 485/535 nm, respectively, and quantified against a standard curve of Rho-123. The fluorescence of the cell lysates was corrected for autofluorescence of untreated cells. For uptake experiments, where the inhibitor was dissolved in ethanol (for example, CsA), fluorescence for ethanol was also corrected. The uptake was normalized to the protein content of cells. Protein content of the samples was measured by the method of Bradford with bovine serum albumin used as the standard. The results were reported as total Rho-123 uptake per milligram of protein. Nonspecific binding was subtracted from overall uptake values obtained.

For energy dependence studies, cells were preincubated for 30 minutes with 1 mM concentrations of metabolic inhibitors such as ouabain (an inhibitor of Na+/K+-adenosine triphosphatase [ATPase]) and sodium azide. Uptake was then performed as described previously with 50 μM Rho-123 in DPBS in presence of inhibitors.

Transport Experiments

Transport of [3H]-CsA across rPCECs and SIRC cells was performed according to a method previously established in our laboratory. Briefly, cells grown in clear polyester membranes (pore size, 0.4 μm) were washed twice with DPBS (pH 7.4) at 37°C. Transepithelial electrical resistance (TEER) of both cell types was measured to elucidate tight junction properties. TEER was measured with a commercial system (EVOM Chopstick Electrodes; World Precision Instruments, Sarasota, FL). Cell layers had TEER values ranging from 150 to 250 Ω · cm². rPCECs with TEER of 200 Ω · cm² or more were used for transport studies. Cells were then mounted on a diffusion apparatus (Side-by-Side; Crown Glass Co., Somerfield, NJ) maintained at 34°C (by constant water supply). [3H]-CsA solution (3 mL, 0.5 μCi/mL) was added to the apical side (donor compartment). In the other half-chamber (receptor compartment), 3 mL of DPBS was added to maintain the hydrostatic pressure in both the half-chambers. The chambers were continuously stirred with magnetic stirrer bars to minimize the aqueous boundary layer. Sink conditions were maintained throughout the experiment. At specified time points, 100-μL aliquots were removed from the receiver chamber and replaced with an equal volume of DPBS. Samples were transferred to scintillation vials containing 5 mL scintillation cocktail, and its radioactivity was measured. [14C]-mannitol, a paracellular marker, was used simultaneously to assess the integrity of the cell layer for the duration of the experiment.

Functional Expression of P-Glycoprotein in Cornea

Analytical Method and Data Treatment

Radioactivity in the experimental samples was measured by a scintillation counter (model LS 6500; Beckman Instruments Inc., Fullerton, CA). These values were used to estimate the transport rate of Rho-123. The data were analyzed as percent uptake, which was calculated according to the formula:

\[
\text{Percent Uptake} = \left( \frac{\text{Radioactivity in experimental sample}}{\text{Radioactivity in standard sample}} \right) \times 100
\]

The statistical analysis was performed using a two-factor ANOVA. Statistical significance was tested by two-factor ANOVA. *P < 0.05; **P < 0.01.
Table 1. Effect of Various P-gp Substrates on the Accumulation of Rho-123 in rPCECs

<table>
<thead>
<tr>
<th>P-gp Substrate/Inhibitor</th>
<th>Concentration (µM)</th>
<th>Rho-123 Uptake (picomoles/mg protein)</th>
<th>Ratio of Rate of Uptake to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho-123 (C)</td>
<td>50</td>
<td>33.25 ± 8.8</td>
<td>1.0</td>
</tr>
<tr>
<td>CsA</td>
<td>5</td>
<td>63.96 ± 5.47</td>
<td>1.92 ± 0.16*</td>
</tr>
<tr>
<td>CsA</td>
<td>10</td>
<td>82.95 ± 6.91</td>
<td>2.49 ± 0.21*</td>
</tr>
<tr>
<td>Testosterone</td>
<td>100</td>
<td>35.6 ± 8.54</td>
<td>1.07 ± 0.26</td>
</tr>
<tr>
<td>Testosterone</td>
<td>200</td>
<td>75.29 ± 12.86</td>
<td>2.20 ± 0.39*</td>
</tr>
<tr>
<td>Quinidine</td>
<td>250</td>
<td>62.25 ± 9.97</td>
<td>1.87 ± 0.30†</td>
</tr>
<tr>
<td>Verapamil</td>
<td>500</td>
<td>50.1 ± 11.66</td>
<td>1.51 ± 0.35†</td>
</tr>
<tr>
<td>Progesterone</td>
<td>200</td>
<td>47.71 ± 4.98</td>
<td>1.43 ± 0.15†</td>
</tr>
</tbody>
</table>

Control uptake is 33.25 ± 8.8 picomoles/mg protein of Rho-123 alone without inhibitor. C denotes that Rho-125 was used as a control. All uptake are for a period of 3 hours. Data are the mean ± SD of four determinations. Statistical significance was tested with a two-factorial ANOVA.

† P < 0.05.

* P < 0.01.

CA). Apparent permeability (P_app) was calculated with the following equation

$$P_{\text{app}} = \frac{(dC/dt) \cdot (V_c \cdot A \cdot 60)}{C_0 \cdot \Delta V}$$  \hspace{1cm} (1)

where $P_{\text{app}}$ denotes the apparent permeability in centimeters per second. $(dC/dt)$ is the slope of plot of concentration (millimolar) versus time (in minutes); $C_0$ is the initial donor concentration of the drug; $V_c$ is the volume of the receiver chamber and, A is the surface area available for diffusion (in Side-bi-Side [Crown Glass Co.] diffusion chambers the surface area for diffusion is 0.636 cm²).

Dose-dependent inhibition data were fitted to a dose-response relationship calculated by equation 2

$$Y = \min + \frac{\max - \min}{1 + 10^{(\log IC_{50} - x \cdot n)}}$$  \hspace{1cm} (2)

where $IC_{50}$ is the inhibitor concentration at twice the rate of uptake and $H$ is the Hill constant. Data were fitted to equation 2 with a transformed nonlinear regression curve analysis program (Prism, version 3.03; GraphPad, San Diego, CA).

Design of Conserved Primers and Reverse Transcriptase–Polymerase Chain Reaction

Corneal epithelial tissues (rabbit and human) and cultured cells were collected and snap frozen in liquid nitrogen. Total RNA was extracted from these tissues and cells by a standard protocol. Briefly, the cells and tissue were taken, and 800 µL of Tri-reagent LS (Molecular Research Center, Inc., Cincinnati, OH) was added. The cells and tissue were homogenized and transferred to Eppendorf tubes (Fremont, CA). RNA was extracted by the phenol-CHCl₃-isopropranol method, purified, and dissolved in 20 µL of RNase–Dnase–free water.

The cDNA sequence for rabbit Pgp is not known, because rabbit P-gp has not yet been cloned. Because cDNA sequences were unavailable, we compared cDNAs from species which have high sequence homology in their protein structure. Three species were chosen for sequence alignment and designing of primers (for rabbit MDR1) from the conserved region of the cDNA. Human (Homo sapiens), mouse (Mus musculus), and rat (Rattus norvegicus) showed 85% homology in the P-gp protein structure. Human MDR1 primers were designed from human MDR1 cDNA (GenBank Accession No: NM_000927; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information [NCBI], Bethesda, MD). The forward and reverse primer designed for human MDR1 was 5'-TCA CCA AGC GGC TCC GAT ACA T-3' and 5'-CCC GGC TGT TGT CTC CAT AGG C-3', respectively. These primers correspond to the nucleotide position 2351-3394. RT-PCR was performed based on the method of Sugawara et al. with modifications using 1 µg of total RNA. RT-PCR was performed with a kit (GeneAmp RNA PCR Kit; Applied Biosystems, Foster City, CA). The conditions for reverse transcription were denaturation of the template RNA for 10 minutes at 70°C and reverse transcription for 60 minutes at 42°C. The conditions for PCR amplification were denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C and extension for 1 minute at 72°C for 37 cycles, and a final extension for 10 minutes at 72°C. The resultant PCR product (~1065 bp) was subcloned in a vector (p-GEM-T-Easy; Promega) and the vector was grown in competent DH5α Escherichia coli cells. The plasmid was purified with a kit (Plasmid Purification Kit; Qiagen Inc., Valencia, CA) and digested with EcoRI to release the cloned insert. Sequencing by the dideoxynucleotide chain termination method was performed by terminator cycle sequencing (TaQ Dye-Deoxy; Applied Biosystems) with an automated sequencer (model 377 Primer; Applied Biosystems). Sequencing was performed from both T7 and SP6 promoter regions to confirm the molecular identity. The sequence was analyzed using a sequence analysis software (GCG, version 10; Genetics Computer Group, Inc., Madison, WI).

Computer Analysis

Database searches and nucleotide sequence homology was performed with basic local alignment tool (BLAST) provided in the public domain by NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple nucleotide sequence alignment was made by ClustalW (1.81) provided in the public domain by European Bioinformatics Institute (EMBL-EBI; http://www.ebi.ac.uk/clustalw/).

Statistical Analysis

All experiments were conducted at least in triplicate and the results expressed as the mean ± SD. IC₅₀ (concentration needed to increase the uptake or inhibit the efflux by 50%) is expressed as the mean ± SE. Statistical significance testing was performed with a two-factor analysis of variance (ANOVA; Statgraphics Plus, ver. 5.1; Manugistics, Inc., Rockville, MD). A difference between mean values was considered significant at P ≤ 0.05. The Fisher least-significance-difference (LSD) method was used to discriminate among the means.

Results

Uptake of Rho-123 in the Presence of Specific Inhibitors

Time-dependent accumulation of Rho-123 in the presence of CsA was investigated to explore the possibility that P-gp may require a longer time to show a more pronounced effect. Uptake of Rho-123 (50 µM) across SIRC cells and rPCECs was investigated at 1, 2, and 3 hours in the presence of 5 µM CsA.
An increase in Rho-123 uptake was observed after 1 hour, and it increased linearly for 3 hours. The greatest increase in Rho-123 uptake was observed at 3 hours of incubation in both the cell types. Control uptake of Rho-123 in rPCECs and SIRC cells was found to be 33.25 ± 8.8 and 14.83 ± 2.87 pmol/mg protein, respectively, in 3 hours. Low control Rho-123 uptake in SIRC cells might suggest the presence of a higher amount of P-gp in the cell line than in rPCECs. Testosterone is known to be a potent and pure inhibitor. Accumulation of Rho-123 in both rPCECs and SIRC cells was not altered significantly in the presence of 100 μM testosterone, thereby suggesting that a major amount of P-gp is inhibited by 200 μM testosterone (Fig. 2). Because these data suggest the existence of an active P-gp efflux pump on the corneal epithelium, we investigated the effect of a series of P-gp substrates on the uptake of Rho-123 (Table 1). Besides CsA and testosterone, we used three well-known P-gp substrates: quinidine, verapamil, and progesterone. CsA (10 μM) showed maximum inhibition followed by testosterone (200 μM). Uptake of Rho-123 increased by a factor of 2.20 (200 μM testosterone) to a maximum of 2.49 (10 μM CsA).

**Dose-Dependent Inhibition of Rho-123 Uptake in rPCECs**

To investigate the nature of the inhibitor and its potency as an inhibitor, dose-dependent inhibition of Rho-123 uptake was performed. At a fixed 50-μM Rho-123 concentration, two inhibitors were studied at various concentrations. The inhibitors selected for this study were CsA and testosterone. Our previous results had suggested that Rho-123 uptake was inhibited in both cell types at low concentrations. As seen in Figure 3, both CsA and testosterone inhibited Rho-123 uptake in a dose-dependent manner. IC50 was calculated for both inhibitors. The data were fitted to a modified log [dose]-response curve fit to yield IC50 values. IC50 values for CsA and testosterone were calculated as 2.01 ± 0.16 and 106.2 ± 3.61 μM, respectively. In both cases, the Hill factor was set at 1.

**Transport of CsA into rPCECs**

Transport experiments were conducted for a period of 120 minutes with [3H]-CsA. The morphology of rPCECs suggested that rPCECs do not form tight junctions (reflected by low TEER of 150–250 Ω·cm²). Rho-123, a small and water-soluble permeant, could not be used as a model substrate to delineate the transport characteristics of the efflux pump. Radiolabeled CsA was chosen, because CsA is a large lipophilic molecule (Mw 1209) and crosses all membrane pathways. [3H]-CsA was used at a concentration of 0.5 μCi/mL. Flux and Papp were calculated from a plot of the cumulative amount of CsA transported as a function of time. The integrity of the rPCEC layer was not compromised during transport, as was evident by the [3H]-mannitol flux (~1.5%/h). There was no polarity in [3H]-mannitol transport, suggesting that mannitol is indeed transported by a paracellular pathway (data not shown). The basolateral-to-apical (B→A) transport of CsA was significantly higher than the apical-to-basolateral transport (A→B; Fig. 4A). The B→A and A→B permeabilities were found to be 5.97 ± 0.48 × 10⁻⁶ cm/s and 1.71 ± 0.12 × 10⁻⁶ cm/s, respectively. In the presence of 500 μM of verapamil (inhibitor) both B→A and A→B permeabilities became equal. The B→A and A→B permeabilities in presence of 500 μM verapamil were 3.74 ± 0.45 × 10⁻⁶ and 3.96 ± 0.40 × 10⁻⁶ cm/s, respectively (Fig. 4B).

**Multiefflux Pathways for Rho-123**

Rho-123 is a lipophilic cation that is thought to be a substrate for the organic cation transport (OCT) system. We therefore investigated the effect of OCT inhibitors on Rho-123 uptake. The OCT inhibitors MPP⁺ and TEA did not change Rho-123 uptake when used between 1 μM and 1 mM (Fig. 5). In addition, preliminary data suggested absence of any other transport processes (BCRP1, LRP, e.g.) that may be involved in the uptake of Rho-123, in both rPCECs and SIRC cells. These data suggest that Rho-123 efflux and transport across rPCEC and SIRC cell layers are primarily affected by P-gp and not by any other carrier-mediated transport processes.
Western Blot Analysis

Expression of P-gp was determined with anti-Pgp monoclonal antibody by a colorimetric amplification method. Figure 6 depicts the immunoprecipitated blot in which the monoclonal antibody reacted with the Pgp, as identified by a dark band at 170 kDa. The result shows that P-gp was localized in the epithelial layers of the cornea and was expressed on the rPCECs.

RT-PCR and Protein Translation

PCR products were analyzed by gel electrophoresis on 0.8% agarose. Human MDR1 primers were obtained from human MDR1 cDNA reported in GenBank. MDR1 cDNA sequences from human, mouse, and rat were aligned according to their sequences, and those segments that are conserved through the species were chosen as sites for development of rabbit specific primers. This study was designed in the hope that the same sequences could identify a similar gene in rabbit. The forward and reverse primers designed for rabbit MDR1 were 5'-CGG ACC ACC ATT GTG ATA GC-3' and 5'-GGT CGG GTG GGA TAG TTG AA-3', respectively. PCR amplifications of specific rabbit and human MDR1 sequences were performed. When rPCEC, SIRC, and intact rabbit corneal RNA was PCR amplified,
all yielded a single product of \(\sim 1100\) bp (Fig. 7). PCR from human cornea yielded a PCR product that was 1065 bp, and when a cloned insert was matched with known sequences, it showed 100% homology with previously reported human MDR1. This result clearly provides evidence that P-gp is present in the human cornea. The \(\sim 1100\) bp fragment was subcloned in a vector (pGEM-T-Easy; Promega) and grown in competent DH5\(\alpha\) E. coli cells. The cloned insert was sequenced from both T7 and SP6 promoter regions and the sequence matched. The final protein sequence was obtained using Protein BLAST and the resultant amino acid sequence matched with any known sequences in GenBank. The sequence match showed more than 89% homology with human MDR1 protein. These protein sequence data suggest that rabbit corneal epithelial cells and cell lines express P-gp at the molecular level that is 89% similar to human P-gp.

**DISCUSSION**

Rho-123 uptake in rPCECs in the presence of various inhibitors of P-gp was investigated. CsA was found to be the strongest among the inhibitors, increasing uptake almost 2.5-fold. Time-dependent uptake of Rho-123 in the presence of CsA and testosterone suggests that P-gp may require a longer time to show more pronounced effect. Inhibition of uptake in 1 hour was not significantly different from control. However uptake of Rho-123 increased significantly in 3 hours. That CsA, a selective and potent P-gp inhibitor, inhibited Rho-123 and increased accumulation is enough evidence that P-gp is present and functioning in these cells. Our results are in agreement with the identification of P-gp in Calu-3 cells.\(^5\)\(^9\) Increased accumulation of Rho-123 has been observed in bovine brain microvesSEL capillary endothelial cells (BBMECs)\(^6\) in the presence of CsA. In our study, CsA was found to be a very potent inhibitor (IC\(_{50}\) \(\sim 2\) \(\mu\)M). Testosterone was not potent at submicromolar concentrations, but it did increase uptake 2.2-fold at 200 \(\mu\)M concentration. P-gp requires energy for the efflux process. Sodium azide, a metabolic poison, inhibited P-gp-mediated Rho-123 efflux. Rho-123 uptake was increased 2.7-fold in the presence of 1 mM NaN\(_3\) (Fig. 8). The ability of sodium azide to inhibit Rho-123 efflux further reconfirms that the efflux occurs by an energy-dependent pathway.

**FIGURE 5.** Effect of OCT inhibitors on Rho-123 uptake in rPCECs. All uptake was performed for 3 hours. Concentration used for MPP\(^+\) and TEA was 1 \(\mu\)M to 1 mM. Statistical significance was tested by two-factor ANOVA. *\(P < 0.01\).

**FIGURE 6.** Western blot analysis of P-gp expression in rPCECs and SIRC cells. The proteins were separated on a 7.5% Tris-glycine gels and transblotted onto a nitrocellulose membrane. They were stained with anti-P-gp monoclonal antibodies. Lanes 1 and 2: membrane fractions from rPCECs and SIRC cells, respectively. Right: molecular weight marker.
Transport of \(^{3}H\)-CsA across rPCEC layers confirms the polarized efflux characteristics of P-gp. In absence of any inhibitor B\(\rightarrow\)A permeability was 3.5-fold higher than A\(\rightarrow\)B permeability. This suggests clearly that P-gp is functional and present in the corneal epithelium. Morphologic studies have shown that rPCECs form multiple cells layers but do not form well-defined tight junctions (data not shown). This is reflected in its low TEER value. The cell layers do not exhibit defined desmosome formation, and there is little zona occludens protein between cells. These few tight junctions are not capable of restricting small molecules such as Rho-123, which, merely because of its size, would permeate paracellularly. Bulky and extremely lipophilic compounds such as CsA (which transports transcellularly) are thus the only substrate of choice.

Transport of Rho-123 in intact rabbit cornea was also performed to identify its function in intact cornea. Unfortunately, because of the presence of the hydrophilic stromal layer Rho-123 molecules accumulated in the stroma and were unable to cross the epithelial layers. Also, this becomes even more complex, because the cornea is multilayered. Therefore, the knowledge of subcellular localization and exact amounts of P-gp in various cell layers, is a prerequisite for developing a model for corneal transport; such studies are currently in progress in our laboratory.

Human MDR1 has been cloned and studied in detail. However, there is no report of cloning of rabbit MDR1. Thus, we looked into making rabbit-specific primers from known nucleotide sequences of human, mouse, and rat MDR1. Conserved regions in cDNA sequences in these three species were chosen because we were hopeful that these regions would not undergo evolutionary changes. These assumptions turned out to be correct when RT-PCR studies with these conserved primers yielded a product with very high sequence homology with known human MDR1. This product was cloned in a vector and grown in E. Coli cells. The cloned insert was sequenced. The amino acid sequence of the fragment protein (sequence deduced using Protein BLAST) was found to have a nearly 89% sequence match with human MDR1. Tissue distribution of human MDR1 has been reported, determined by Northern blot analysis. However, it did not include any ocular tissues. With the partial sequence and its homology known, it would be easy to clone the entire rabbit MDR1 gene.

Multidrug resistance in epithelial cells is associated with the overexpression of certain MDR proteins. Numerous drug efflux pumps (e.g. P-gp, MRP1) are known to limit absorption of therapeutic agents. Drug efflux is a highly complex process because its mechanism is poorly understood. Previously, presence of this efflux pump was evidenced while studying CsA transport across cultured corneal epithelial cells. It was thought that P-gp is causing the drug efflux and that CsA-binding protein, cyclophilin is expressed in the corneal cells. In this study, we have provided molecular characterization of the existence of P-gp in cultured corneal cells and intact cornea. We have shown, beyond doubt, that functional P-gp is expressed in cornea. This is the first report of the existence of P-gp in human cornea and a partial protein structure of rabbit P-gp, which is not yet cloned. Current knowledge and understanding of the existence of P-gp in cornea and other ocular tissues will change drug delivery strategies to increase ocular bioavailability. In the past, low ocular bioavailability was attributed to the inability of the drug to cross the lipoidal membrane and not to any efflux pump that may be playing an important role in preventing drug permeation. It is now clear that drugs that are poorly absorbed may be effluxed by P-gp present in the cornea.

In conclusion, this study demonstrates functional evidence of the existence of P-gp in rabbit cornea. Molecular studies and protein sequence deductions have shown that rabbit MDR1 and human MDR1 share a high sequence homology (89%), which is very much expected because the sequences of the genes are conserved within the mammalian system. rPCECs could be used as an in vitro model for ocular bioavailability studies involving drugs and compounds that are P-gp substrates. It would be a valuable tool for screening such compounds. In the future, cloning and expression of rabbit MDR1 will help us gain valuable insights into the characteristics of its function in intact cornea.
this efflux pump. Drug delivery to the cornea and to the inner chambers of the eye will certainly change with this new knowledge, and targeted drug delivery using a produg approach to bypass this efflux protein could be imminent.

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References


