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Research Paper

Multidrug Resistance Protein 1 (MRP1) in Rabbit Conjunctival Epithelial Cells: Its Effect on Drug Efflux and Its Regulation by Adenoviral Infection

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Purpose. To evaluate the expression, localization, function, and regulation of multidrug resistance protein (MRP1) in rabbit conjunctival epithelial cells (RCEC).
Materials and Methods. MRP1 gene expression in RCEC was determined by reverse transcription-polymerase chain reaction (RT-PCR), and MRP1 protein expression and its localization were determined by Western blot analysis and immunofluorescence using an anti-MRP1 monoclonal antibody, MRPr1. The effect of MRP1 on the transport and uptake of fluorescein was evaluated in RCEC grown on Transwell filters. Moreover, the effect of adenovirus type 5 (Ad5)-infected RCEC, and cytokines (Interleukin 1 (IL-1), IL-6, and tumor necrosis factor alpha (TNFα)) on MRP1 expression and leukotriene C_4 (LTC_4) uptake were investigated.
Results. A 652 bp RT-PCR product from rabbit conjunctiva showed a 87% homology to human MRP1. Immunostaining with MRPr1 revealed a predominant basolateral localization of MRP1 in RCEC. Uptake of fluorescein, a MRP1 substrate, was increased (203–290%) in the presence of uricosuric drug probenecid at 100 μM, anti-inflammatory drug indomethacin at 10 μM and diclofenac, flurbiprofen, and ofloxacin at 1 mM, and by ATP depletion, but not influenced by the depletion of GSH, and the presence of antiviral cidofovir and anti-inflammatory drug cromolyn and prednisolone. Apical-to-basolateral facilitated transport of LTC_4 was abolished in the presence of probenecid. Western blot analysis with MRPr1 revealed a distinct band at ~190 kDa for freshly isolated and cultured RCEC. Both Ad5 and cytokines (IL-1, IL-6, and TNF-α) up-regulated MRP1 expression, thereby reducing LTC_4 uptake.
Conclusions. MRP1 appears to be primarily localized in the basolateral membrane of RCEC and function in the efflux of certain organic anions and inflammatory factors out of cells from the basolateral membrane. The upregulation in the expression of MRP1 by Ad5-infection and cytokines suggests a role of MRP1 in the transport of inflammatory factors during ocular inflammation. Supported by NIH grants EY12578, EY10421, and EY12356.

KEY WORDS: adenoviral infection; conjunctival epithelial cell; cytokine; MRP1; regulation.

INTRODUCTION

Multidrug resistance not only represents an obstacle in anti-cancer therapies, but also leads to decreased drug absorption into various tissues. Multidrug resistance can be conferred by expression of either P-gp which is a 170 kDa protein encoded by MDR1 gene (1) or MRP1 which is a 190 kDa protein encoded by MRP1 gene (2). These efflux systems belong to the ATP-binding cassette (ABC) superfamily of membrane transporter proteins and function to pump xenobiotics or their conjugates out of cells (3). P-gp and MRP1 are not only over-expressed in cancer cells, but also are expressed in normal tissues such as intestinal, hepatic, renal epithelial cells, blood brain barrier, adrenal gland (4,5) where they play a role in the protection against various xenobiotics. In the eye, P-gp has been found in the retina, iris (6), cornea (7), and conjunctiva (8). P-gp appears to typically localize in the apical membrane of polarized cells (4), whereas MRP1 localizes in basolateral membrane of the epithelial cells in kidney, intestine, and hepatocytes (9–11), but apical membrane of brain capillary endothelial cells (12). Both proteins have a broad substrate specificity which is partly overlapping, such as anticancer drugs daunorubicin, doxorubicin, etoposide, and vincristine. P-gp appears to prefer neutral and/or positively charged lipophilic molecules, including lipophilic drugs such as cyclosporin A (CsA), vinblastine, etoposide, cimetidine, and celiprolol (13), whereas MRP1 has been identified as a transporter of organic anions and glutathione S-conjugates (14). Transmembrane

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transport of GSH conjugates indicates that MRP1 functions as detoxification protein to eliminate toxic substrates from the cells. A unique feature of translocation mechanism is that MRP1-mediated transport is inhibited by depletion of intracellular glutathione (GSH). Its ability of pumping out oxidized GSH or GSSG as a potential physiological substrate raises the possibility of a role of MRP1 in cellular defense against oxidative stress and perhaps also the maintenance of intracellular redox potential (2).

Both P-gp and MRP1 have been reported to participate in host immune response. NK cells and $CD8^+$ T cells have high P-gp expression. Knockout of MDR1 abolished cytolytic function of the NK cells *in vitro* (15). Functional P-gp may serve additionally to protect these important immune cells in the hostile environment of an immune response, as indicated by the involvement of P-gp in the transport of cytokines, such as IL-1β, IL-2, IL-4, IFN-γ, and TNF-α out of activated lymphocytes into the surrounding medium (16). In MRP1-deficient mice, the response to an inflammatory stimulus was impaired. This was attributed to a decreased secretion of leukotriene C4 (LTC_4), an inflammatory mediator, thus supporting the hypothesis that MRP1 has a physiological role as a transporter of LTC_4 (17).

The conjunctiva is the first tissue across which topical drugs must pass in order to reach the underlying tissues via non-cornea route (18). We have reported that P-gp exists in the rabbit conjunctival epithelial cells and impairs the absorption of immusuppression drug CsA and β-blocker propranolol (8,19). From ocular drug delivery point of view, it is important to understand whether MRP1 is also present in the conjunctiva, its regulation, and its effect on drug transport. Moreover, it is of interest to investigate the roles of MRP1 in ocular infection and inflammation. In this study, we evaluated the expression and localization of MRP1 in rabbit conjunctival epithelial cells at both gene and protein levels and its function on the transport of MRP1 substrates fluorescein, LTC_4, and other ocular drugs as well. The regulation of MRP1 on protein level and its efflux function in RCEC with Ad5 and cytokine (Interleukin 1 (IL-1), IL-6, and tumor necrosis factor alpha (TNFα)) challenge were also investigated.

MATERIALS AND METHODS

Materials

[^{3}H]-Leukotriene C_4 (specific activity, 115 Ci/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA). [^{14}C]-D-Mannitol (specific activity, 56 Ci/mmol) and [G-^{3}H] vincristine sulphate (specific activity, 5.5 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). Fluorescein sodium, indomethacin, probenecid, sodium azide, buthionine sulfoximine (BSO), diclofenac, ofloxacin, flurbiprofen, cromolyn, prednisolone, GSH, progesterone, verapamil, rhodamine123, 2,4-dinitrophenol (2,4-DNP), tetraethylammonium (TEA) bromide, and guanidine were purchased from Sigma Chemical Co. (St. Louis, MO). CsA was obtained from Sandoz Pharmaceuticals (Basel, Switzerland). Cidofovir was a gift from Gilead Sciences (Foster City, CA). Cell culture media and supplies were obtained from Gibco (Grand Island, NY). PC-1 (a serum-free, low-protein, defined medium) culture medium was purchased from BioWhittaker (Walkersville, MD). Transwell filters (6.5 mm diameter, 0.4 μm pore size) were obtained from Costar (Cambridge, MA). MRPr1 mAb was purchased from Kamiya Biomedical Company (Seattle, WA). FITC-conjugated donkey anti-rat IgG and peroxidase-conjugated AffiniPure donkey anti-rat IgG were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Mouse IL-1, IL-6, and TNF-α were purchased from PharMingen (San Diego, CA). Male Dutch-belted pigmented rabbits, weighing 2.0–2.5 kg, were obtained from American Rabbitry (Los Angeles, CA), and all animals were handled in accordance with the Guiding Principles in the Care and Use of Animals (Department of Health, Education, and Welfare, NIH; Publication 80-23, and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research).

Primary Culture of Rabbit Conjunctival Epithelial Cells

Rabbit conjunctival epithelial cells (RCEC) were harvested using a protocol developed by Yang *et al.* (20). Briefly, following excision, the conjunctiva was washed in ice-cold Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution and treated with 0.2% protease (type XIV, 11.2 units/ml) for 60 min at 37°C in 95% air/5% CO_2 to dissociate the cells. The isolated cells were treated with S-MEM containing 10% FBS and 1 mg/ml deoxyribonuclease (DNase I, 2,300 Kunitz units/ml) to stop protease reaction and centrifuged at $100 \times g$ for 10 min at room temperature. The resuspended cells were filtered through a 40 μM cell strainer and pelleted. The final cell pellet was resuspended in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 100 U/ml penicillin-streptomycin, 0.5% gentamicin, 0.4% fungizone, 2 mM L-glutamine, 1% ITS^+ (insulin 6.5 μg/ml, transferrin 6.5 μg/ml, selenious acid 6.5 ng/ml, BSA 1.25 mg/ml, and linoleic acid 5.35 mg/ml), 30 μg/ml bovine pituitary extract (BPE), 1 μM hydrocortisone, and 1 ng/ml epidermal growth factor (EGF). These cells were seeded at a density of 1.2×10^6 cells/cm^2 on Transwell inserts pre-coated with Rat Tail Collagen I (0.077 mg/ml), and cultured in 5% CO_2 and 95% air at 37°C. From day 2 onward, the growth medium was changed to PC-1 growth medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 0.5% gentamicin and 0.4% fungizone. Cells were then switched to an air-interface (i.e., nominally fluid-free on the apical surface of the cell layers from that day onward) on day 4 onward, unless otherwise indicated. The transepithelial electrical resistance (TEER) in kΩ.cm^2 and potential difference (PD, apical negative) in mV were measured with an EVOM epithelial voltohmeter (World Precision Instruments, Sarasota, FL). Confluent RCEC layers with the resistance above 0.9 kΩ.cm^2 were used in this study.

Molecular and Cellular Characterization of MRP1

Reverse Transcription-Polymerase Chain Reactions

Total RNA extracted from freshly isolated rabbit conjunctival epithelial cells and cultured Caco-2 cells by Trizol® Reagent (Gibco BRL, Grand Island, NY) was used for RT-PCR. First strand cDNA was synthesized with

oligo(dT) primer and Superscript II RT (Gibco BRL, Grand Island, NY). For the PCR step, three paired restricted primers were designed from the highly conserved amino acid sequences identified by multiple sequence alignment of the human *MRP1* (5,927 bp) and mouse *Mrp1* cDNA (4,587 bp), which were to amplify a 652-base pair (bp) (the sense primer corresponded to human *MRP1* cDNA residues 889-915, 5′-GCT ACC GCC AGC CCC TGG-3′; the antisense primer corresponded to residues 1522-1539, 5′-GGG GCT GAC CAG ATC ATG-3′); a 538 bp (the sense primer corresponded to human *MRP1* cDNA residues 1786-1803, 5′-CAT CAG GCA GGA GGA GCT-3′; the antisense primer corresponded to residues 2307-2323, 5′-GGC CAC GGA GCC CTT GA-3′), and a 351 bp (the sense primer corresponded to human *MRP1* cDNA residues 4267-4284, 5′-CAT CGC CAA GAT CGG CCT-3′; the antisense primer corresponded to residues 4601-4617, 5′-GTC CGG ATG GTG GAC TG-3′) products. PCR was performed in a thermal cycler (Perkin-Elmer, Norwalk, CT) set to the following conditions: 94°C for 4 min, 1 cycle; 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, 30 cycles; 72°C for 10 min, 1 cycle. The PCR products by using Primer 652 were electrophoresed at 80 mV through a 1.5% agarose gel, and size-selected DNA fragments were extracted and subcloned into the pGEM®-T Easy Vector System (Promega, Madison, WI) and sent for sequencing at Genemed Synthesis, Inc. (South San Francisco, CA).

Immunohistochemical Detection of MRP1 in Cultured RCEC and Excised Conjunctiva

The excised conjunctiva was fixed with 4% paraformaldehyde and infiltrated with 30% sucrose solution. The tissue was then frozen in liquid nitrogen and cut into 8 μM sections on cryodisc and collected on "superfrost plus" charged slides. After tissue section was permeabilized with 0.5% Triton X-100 in PBS for 15 min, one percent of bovine serum albumin (BSA) was used to block non-specific binding sites for 1 h at room temperature. The section was reacted with primary antibody (MRPr1, 2 μg/ml) and FITC-conjugated donkey anti-rat IgG (1:50) sequentially for 1 h each at room temperature. After washing, the immunofluorescence staining was visualized under confocal microscopy (Zeiss, Germany). The confluent cultured RCECs grown on the filter were directly fixed, permeabilized, blocked, and incubated with primary and secondary antibodies essentially the same way as the treatment for the tissue. Then, the cells with the filter was cut out, mounted on the slide, and viewed under confocal microscopy.

Western Blot Analysis

Western blot was performed with the MRPr1 rat monoclonal antibody for MRP1 (5). Freshly isolated RCEC, positive controls of small intestinal epithelial cells and kidney cortex and RCEC culture control, Ad5-infected, cytokine-treated RCEC cultures, were homogenized in a MSEP (125 mM Mannitol, 40 mM sucrose, 1 mM EDTA-Tris, and 5 mM PIPES-Tris, pH 6.7) buffer containing protease inhibitors in an ice bath. The suspension was centrifuged at $1{,}000 \times g$ for 10 min and the supernatant was transferred into a new tube and centrifuged for 15 min at $367{,}000 \times g$. The pellet was resuspended in MSEP and membrane protein was measured by the DC protein assay (Bio-Rad, Hercules, CA), using BSA as a standard. Twenty μg of cell proteins were electrophoresed on 8% SDS-polyacrylamide gel, and subsequently electrotransferred to a nitrocellulose membrane (Amersham, Downers Grove, IL). The sheet was incubated for one hour in PBS containing 1% BSA, 1% nonfat dry milk, and 0.05% Tween-20, to prevent nonspecific binding of antibodies. Incubation with MRPr1 (1 μg/ml) was conducted at 4°C overnight. Immunoreactivity was visualized with peroxidase-conjugated donkey anti-rat immunoglobulins (1:20,000). Immunoblot procedure based on the enhanced chemiluminescence method (ECL) was performed by exposing the membrane to the ECL detection reagent (Amersham, Downers Grove, IL) for 1 min, followed by immediate exposure to X-ray film. The band density was measured by a program NIH image 1.52.

Efflux Function of MRP1

Fluorescein and LTC_4 Uptake

Prior to each uptake experiment, the cell layers on Transwell inserts were washed and pre-equilibrated 30 min with a pH 7.4 bicarbonated Ringer's solution (BRS) containing 1.8 mM $CaCl_2$, 5.6 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM NaH_2PO_4, 25 mM $NaHCO_3$, 116 mM NaCl, 15 mM HEPES, and 5.5 mM D-glucose at 37°C (300 mOsm/kg water). Given that 1-h uptake of fluorescein in cultured RCEC was spectrofluorometically measurable, this time period was chosen in fluorescein uptake study. To determine whether the transport of fluorescein and some ocular drugs is mediated by MRP1, uptake of fluorescein at 100 μM from basolateral compartment was measured in the absence or presence of MRP1 inhibitors, 100 μM probenecid, 10 μM indomethacin, or ocular organic anion and steroid drugs, cidofovir, diclofenac, ofloxacin, flurbiprofen, cromolyn, and prednisolone, all at 1 mM in basolateral compartment. With the pre-incubation of cells in glucose-free BRS containing 10 mM sodium azide and 5 mM mannitol for 30 min, fluorescein uptake was determined in order to evaluate the ATP-dependent transport process. One-hour uptake was terminated by washing the cell layers successively three times with 100 ml ice-cold BRS. The washed cell layers were solubilized in 1 ml of 0.5% Triton X-100 solution and transferred to 4.5 ml cuvette. Twenty μl of the cell lysate was taken for protein assay using the method of Bradford (21), with bovine serum albumin as a standard. Drug uptake was expressed as amount of drug accumulated per mg of cellular protein over the duration of measurement. The fluorescence intensity of the samples was measured in a spectrofluorometer F-2000 (Hitachi, Tokyo, Japan) at an excitation and an emission wavelength of 488 and 510 nm, respectively.

One-hour uptake of LTC_4 was performed by adding a dosing solution containing [^{3}H] LTC_4 (1.15 μCi/ml, 10 nM) to the basolateral compartment, followed by the same washing and protein assay steps as in fluorescein uptake experiment. The cell lysates were mixed with 5 ml of EconoSafe scintillation cocktail (Research Products International Corp., Mount Prospect, IL) for assay of radioactivity in a liquid scintillation spectrometer (Beckman, Fullerton, CA).

Effect of GSH on Fluorescein Uptake

To examine the effect of GSH on MRP1-mediated fluorescein transport, intracellular glutathione was depleted by addition of BSO (100 μM) to both apical and basolateral compartments. Intracellular non-protein thiol (largely GSH) was measured with 5,5′-dithiobis(-2-nirobenzoic acid) (Ellman's reagent; Boehringer Mannheim) in the supernatant of trichloroacetic acid (TCA) precipitated cells. One-hour fluorescein uptake was evaluated in the cells with 18-h treatment of 100 μM BSO.

Fluorescein and LTC_4 Transport

Transport study was carried out in confluent cell layers on Transwell inserts by addition of a dosing solution containing fluorescein (100 μM), [^{3}H] LTC_4 (1.15 μCi/ml, 10 nM), or a paracellular marker radiolabeled [^{14}C] mannitol (1 μCi/ml, 18 μM), to the apical (0.2 ml, for apical-to-basolateral transport (a-to-b)) or basolateral (0.8 ml, for basolateral-to-apical transport (b-to-a)) compartment in the absence or presence of 1 mM probenecid in both apical and basolateral compartments. At predetermined times for up to 3 h, 0.2 ml of receiver solution from the basolateral side (for a-to-b) or 0.1 ml of receiver solution from the apical side (for b-to-a), was sampled. An equal amount of fresh BRS with or without probenecid was replaced immediately after sampling. The fluorescein samples were placed into a 4.5 ml cuvette with addition of 1 ml of BRS and measured in a spectrofluorometer. The mannitol and LTC_4 samples were placed in a scintillation vial and mixed with 5 ml of EconoSafe scintillation cocktail for assay of radioactivity in a liquid scintillation spectrometer. The time course of accumulation of radioactivity was analyzed for steady-state flux and, in turn, apparent permeability coefficient, as described subsequently.

Vincristine Uptake

To understand the relative contribution of P-gp and MRP1 to total drug efflux in the conjunctiva, one-hour uptake of 200 nM [^{3}H] vincristine, a known substrate of both efflux pumps, was studied under various conditions. Both apical and basolateral compartments of the cell layers were dosed with 200 nM [^{3}H]vincrinstine and one of other agents including a P-gp and MRP1 substrate CsA (10 μM); P-gp substrates: quinidine (100 μM) and rhodamine 123 (100 μM); MRP1 substrates: indomethacin (10 μM) and probenecid (100 μM); organic cations: TEA (100 μM) and guanidine (100 μM); or organic anions: lactic acid (100 μM), nicotinic acid (100 μM), and cidofovir (100 μM). For BSO treatment group, BSO at 100 μM was used to treat the cells overnight (18 h) before uptake experiments.

The Regulation of MRP1 by Ad5 Infection and Cytokines

On Day 4 post-seeding, RCEC were inoculated with Ad5 at a multiplicity of infection of 10 from apical chamber at 37°C in 5% CO_2 incubator for 3 h. Then, the inoculum was removed, and the cell layers were rinsed three times with medium, after which growth medium was added and the cells

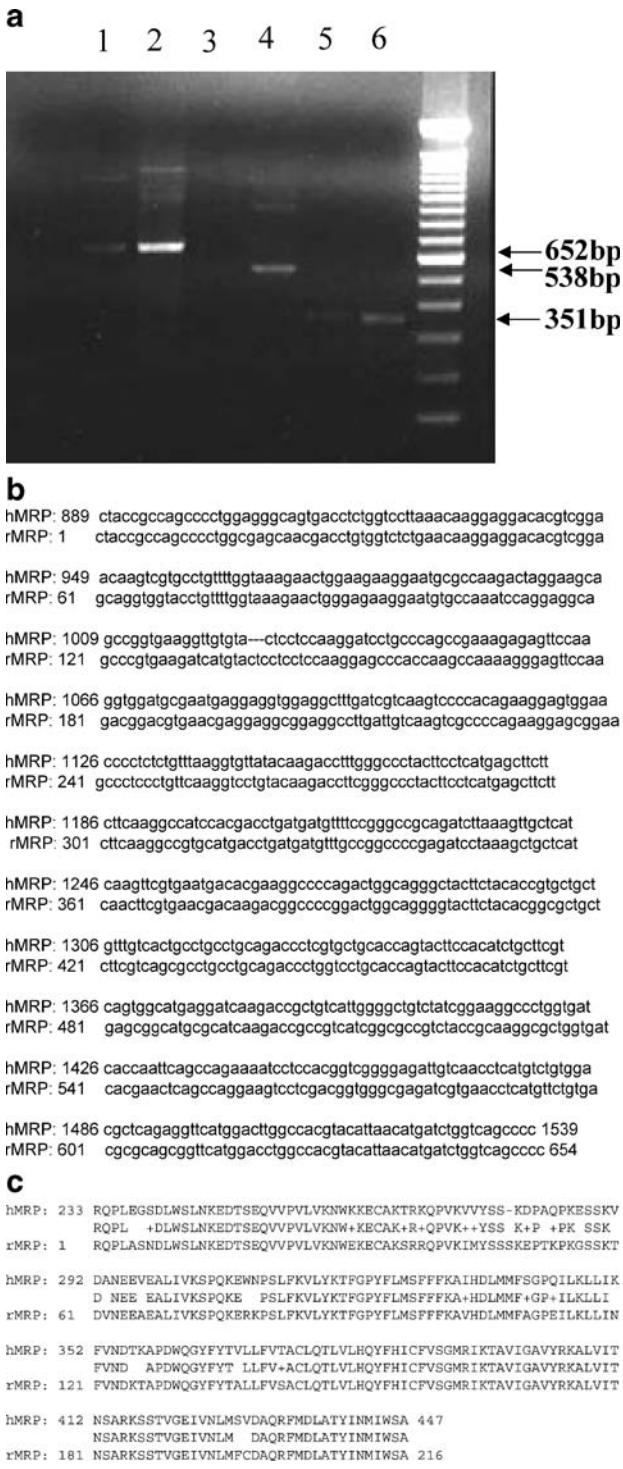

Fig. 1. **a** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression by genes encoding MRP1 in the RCEC and Caco-2 cells. Lanes 1, 3, 5 are Caco-2 cells, and lanes 2, 4, 6 are RCEC. Lanes 1 and 2 are MRP1 specific RT-PCR product of 652 bp, Lanes 3 and 4 are MRP1 specific RT-PCR products of 538 bp, and Lanes 5 and 6 are MRP1 specific RT-PCR product of 351 bp. **b** Nucleotide sequence alignment of RT-PCR product targeting rabbit MRP1 with human MRP1. hMRP1: Human MRP1; rMRP1: Rabbit MRP1. Identities = 554/654 (84%). **c** Amino acid sequence alignment of RT-PCR product targeting rabbit MRP1 with human MRP1. hMRP1 = Human MRP1, rMRP1 = Rabbit MRP1. Identities = 188/216 (87%).

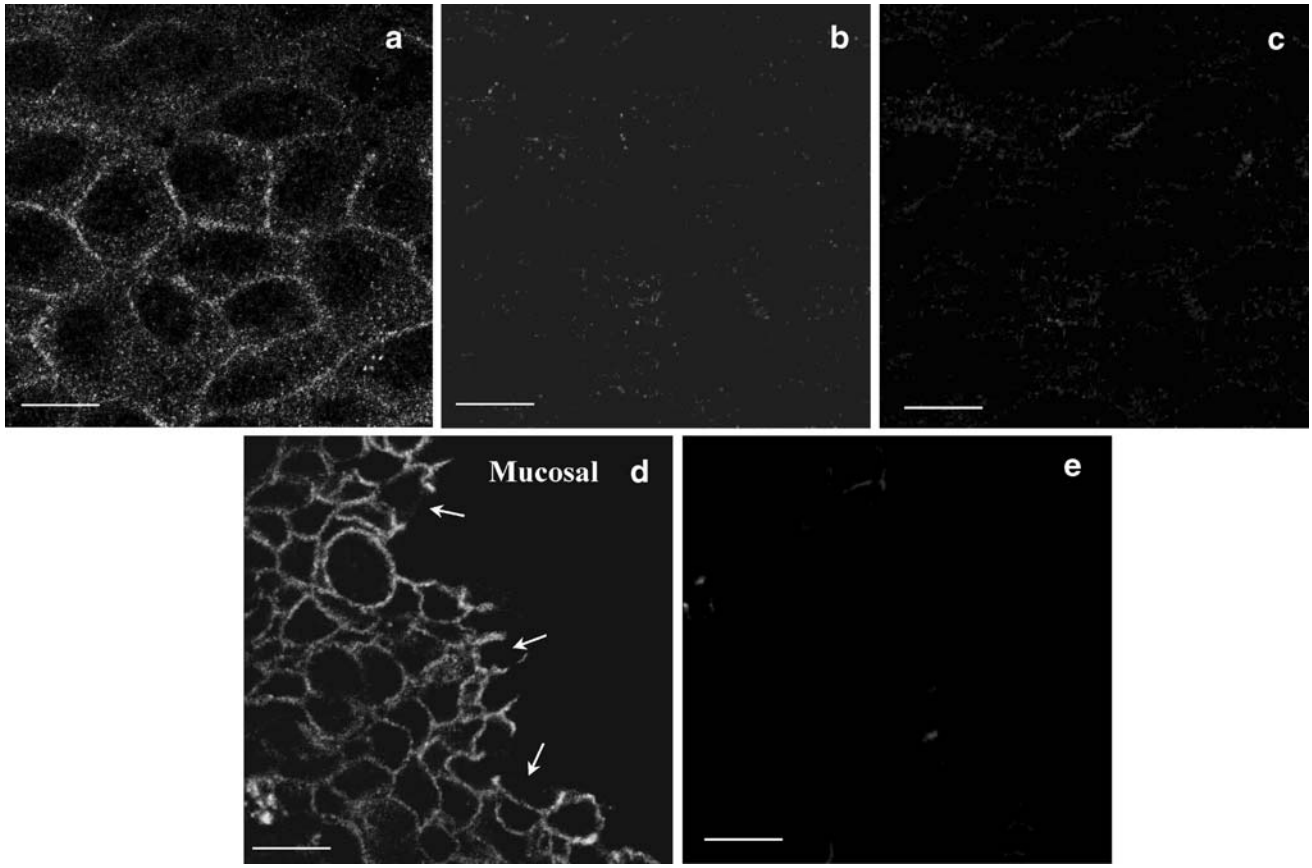

Fig. 2. Immunolocalization of MRP1 in cultured RCEC and rabbit conjunctiva. Panels **a** and **b** are the immunofluorescence images observed on the basolateral and apical membrane of cultured RCEC, respectively, with MRPr1 mAb treatment by confocal laser-scanning microscopy. Panel **c** is the immunofluorescence image found without MRPr1 mAb, but with secondary antibody alone. Panels **d** and **e** are images of rabbit conjunctiva with or without MRPr1. *Arrows* point the apical surface of the rabbit conjunctival superficial cell layers. (original magnification 600×). Bar lengths are 10 μM.

were incubated at an incubator. Another set of RCEC was treated with IL-1 (10 ng/ml), IL-6 (100 U/ml), TNF-α (100 U/ml), or the combination of three cytokines. Forty-eight hours after Ad5 inoculation or cytokine treatment, membrane protein obtained from RCECs was used for MRP1 expression measurement, and in parallel, functional study of MRP1 was evaluated by using LTC_4 (10 nM) basolateral uptake.

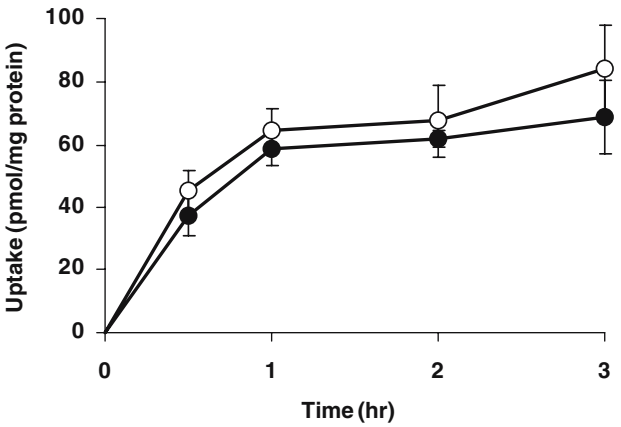

Fig. 3. Time course of fluorescein uptake at 100 μM in cultured rabbit conjunctival epithelial cells. Data are mean ± s.e.m. ($n = 3$). Keys: ○, dosing from apical side; ●, dosing from basolateral side.

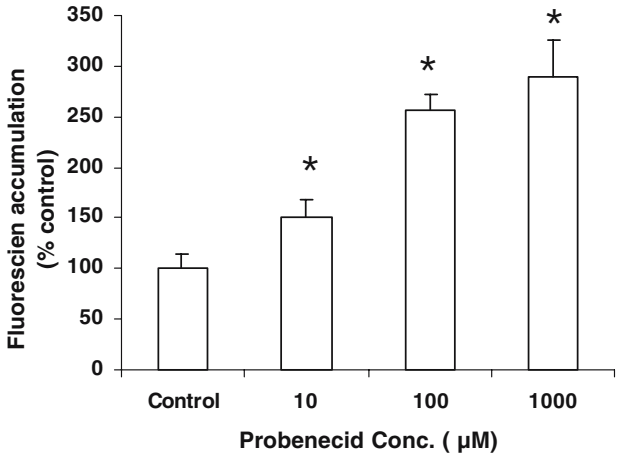

Fig. 4. One-hour uptake of fluorescein (at 100 μM from basolateral compartment) in the presence of probenecid at various concentrations. Data are mean ± s.e.m. ($n = 4$).

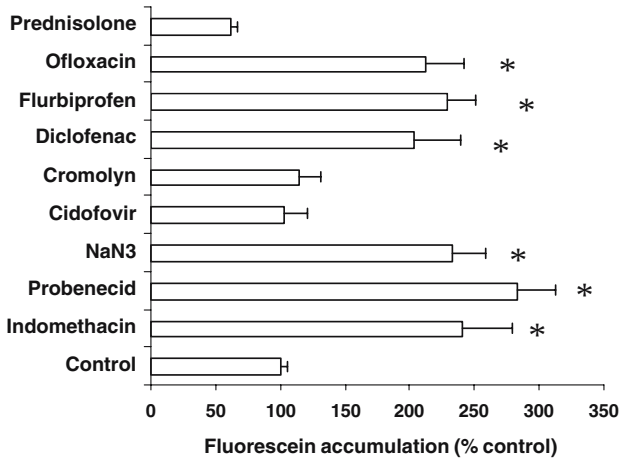

Fig. 5. One-hour uptake of fluorescein (at 100 μM from basolateral compartment) in the presence of MRP1 inhibitors: indomethacin (10 μM) and probenecid (100 μM), organic anions (1 mM): cidofovir, ofloxacin, flurbiprofen, diclofenac and cromolyn, and steroid prednisolone, or with a pretreatment with 10 mM NaN_3/glucose free medium for 30 min. Values are mean ± s.e.m., $n = 3$–4. Asterisks indicate a statistically significant difference from basolateral uptake at 37°C. *Asterisks* indicate a statistically significant difference ($p < 0.05$) from the control.

Data Analysis

The steady-state flux was estimated from the slope of the linear portion of a plot of a cumulative amount of drug appearing in the receiver fluid as a function of time. The apparent permeability coefficient (Papp) of unidirectional fluxes for solutes was estimated by normalizing the flux, dQ/dt (mol/s), against the nominal surface area ($A = 0.33$ cm^2) and initial solute concentration in the donor fluid, C_0 (mol/ml), or $Papp = (dQ/dt)/(A \times C_0)$.

Two-tailed Student's *t* test for unpaired data for two groups or one-way analysis of variance for multiple comparisons was used to determine the significance of difference between means of more than two data groups. Post hoc comparisons were made using Tukey's multiple comparison test to contrast statistical significance among group (≥3) means. $p < 0.05$ was considered as statistically significant.

RESULTS

Molecular and Cellular Characterization of MRP1

Reverse Transcription-Polymerase Chain Reaction

To evaluate *MRP1* expression at gene level in the rabbit conjunctival epithelial cells, RT-PCR was performed using restricted primers designed according to the highly conserved regions of the early, middle, and late portions of cloned human *MRP1* and mouse *Mrp1* cDNAs. As shown in Fig. 1a, this approach yielded products of about 652, 538, and 351 bp, which were of the desired sizes. The PCR product of 652 bp was then subcloned and sequenced. Sequence comparison showed that this RT-PCR product was 84% identical to human *MRP1* in nucleotide sequence (Fig. 1b) and 87% identical in amino acid sequence (Fig. 1c).

Localization of MRP1

Positive staining was observed primarily in the basolateral membrane (Fig. 2a), but not the apical membrane (Fig. 2b) of cultured RCEC in the presence of MRPr1 mAb under confocal microscopy. Membrane staining pattern was not observed in the cells stained with the secondary antibody alone (Fig. 2c). Similarly, the apical surface of the superficial layer of rabbit conjunctiva tissue showed no staining, whereas the basolateral membrane was positively stained (Fig. 2d). Without MRPr1 mAb treatment, no staining was observed in the tissue section (Fig. 2e). Only sporadic intracellular staining was also observed in both cultured RCEC and conjunctiva tissue.

Efflux Function of MRP1

MRP1-mediated Transport and Uptake of Fluorescein and LTC_4 in Cultured RCEC

Papp of a-to-b transport of fluorescein at 100 μM was $2.02 \pm 0.16 \times 10^{-7}$ cm/s, that of b-to-a transport was $1.41 \pm 0.03 \times 10^{-7}$ cm/s, indicating a preferred a-to-b transport in cultured RCEC ($p < 0.05$).

Fluorescein uptake by the cells was time dependent, with the uptake plateauing after 1 hr. There was no significant

Table I. Fluorescein, LTC_4, and Mannitol Transport Across Cultured Rabbit Conjunctival Epithelial Cell Layers

Solutes	Condition	Ratio[a]	Papp (x10^{-7} cm/s)[b]	
			a-to-b	b-to-a
Fluorescein (100 μM)		1.4*	2.02 ± 0.16	1.41 ± 0.03
LTC_4 (10 nM)	Control	1.4*	3.32 ± 0.44	2.33 ± 0.09
	+ 100 μM Probenecid	1.1	2.86 ± 0.08	2.56 ± 0.08
Mannitol (18 μM)		0.9	2.18 ± 0.15	2.45 ± 0.15

[a] a-to-b/b-to-a Papp
[b] mean ± s.e.m. ($n = 3$–6)
*$p < 0.05$

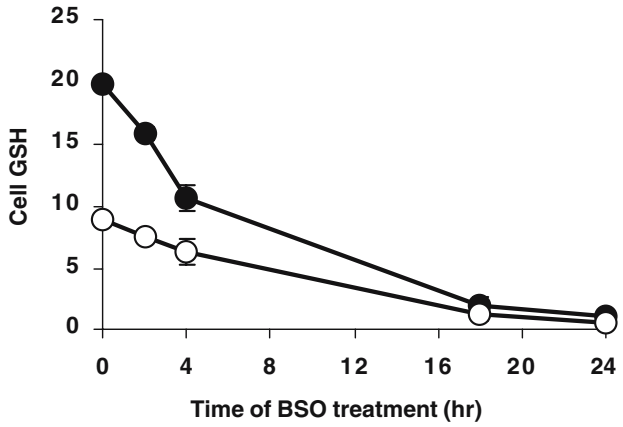

Fig. 6. Time course of glutathione (GSH) in RCEC after treatment with 100 μM BSO. Data are mean ± s.e.m. ($n = 3$). Key: •, nmol/mg protein; ○, nmol/million cells.

difference in fluorescein uptake between apical and basolateral dosing (Fig. 3). Probenecid increased fluorescein uptake in a dose dependent manner—151% at 10 μM, 256% at 100 μM, and 290% at 1,000 μM, respectively, compared to the control (100%) (Fig. 4). In the presence of 10 μM indomethacin, fluorescein uptake from basolateral dosing was increased (240%). Among an ocular antiviral (cidofovir), NSAIDs (diclofenac, ofloxacin, flurbiprofen, and cromolyn), and a steroid anti-inflammatory drug (prednisolone), only diclofenac (203%), ofloxacin (229%), and flurbiprofen (213%) at 1 mM increased fluorescein uptake compared to the control. By pretreating the cells with glucose-free BRS containing 10 mM NaN_3 for 30 min, the fluorescein uptake was increased (233%) (Fig. 5).

Papp of a-to-b transport of LTC_4 at 10 nM across the cultured RCEC was $3.32 \pm 0.44 \times 10^{-7}$ cm/s, that of b-to-a transport was $2.33 \pm 0.09 \times 10^{-7}$ cm/s ($p < 0.05$). In the presence of 1 mM probenecid, the directionality was abolished with Papp of a-to-b transport of $2.86 \pm 0.08 \times 10^{-7}$ cm/s and that of b-to-a transport of $2.56 \pm 0.08 \times 10^{-7}$ cm/s. In contrast, the transport of mannitol, a paracellular marker, did not show directionality with Papp of a-to-b transport of $2.18 \pm 0.15 \times 10^{-7}$ cm/s and that of b-to-a transport of $2.45 \pm 0.12 \times 10^{-7}$ cm/s (Table I).

GSH Depletion and Its Effect on Fluorescein Uptake

GSH concentration in the RCEC was 8.97 ± 0.06 nmol/million cells or 19.84 ± 0.02 nmol/mg protein. Overnight treatment of the RCEC with BSO at 100 μM lowered GSH concentration by 90% (Fig. 6), but fluorescein uptake was not affected (Table II).

Table II. One-hour Fluorescein Uptake and Cell Viability with a Pretreatment of 100 μM BSO for 18 hr

	Fluorescein Uptake pmol/mg Protein	Cellular GSH nmol/million	Cell Viability (%)
Without treatment	55.80 ± 3.22	8.97 ± 0.06	96
With BSO treatment	60.84 ± 6.10	1.15 ± 0.39	94

Values are mean ± s.e.m., $n = 3$–4.

Contribution of P-gp and MRP1 to Vincristine Uptake

To understand the relative contribution of P-gp and MRP1 to total drug efflux, one-hour uptake of 200 nM [^{3}H]vincristine, a known substrate for both efflux pumps, was studied under various conditions. Vincristine uptake was increased in the presence of a P-gp and MRP1 substrate CsA (194%); P-gp substrates: quinidine (154%) and rhodamine (132%); and MRP1 substrates: indomethacin (175%) and probenecid (169%); but not in the presence of organic cations: TEA (87%) and guanidine (90%); and organic anions: lactic acid (86%), nicotinic acid (106%), and cidofovir (126%). BSO overnight treatment also caused significant increase in vincristine uptake (131%) (Fig. 7).

Regulation of MRP1 by Ad5 Infection and Cytokines

MRP1 Protein Expression by Western Blot Analysis

Western blot analysis with MRPr1 mAb revealed a distinct band at ~190 kDa for freshly isolated RCEC and the positive controls rabbit small intestinal epithelial cells and kidney cortex (Fig. 8a). The MRP1 band density was

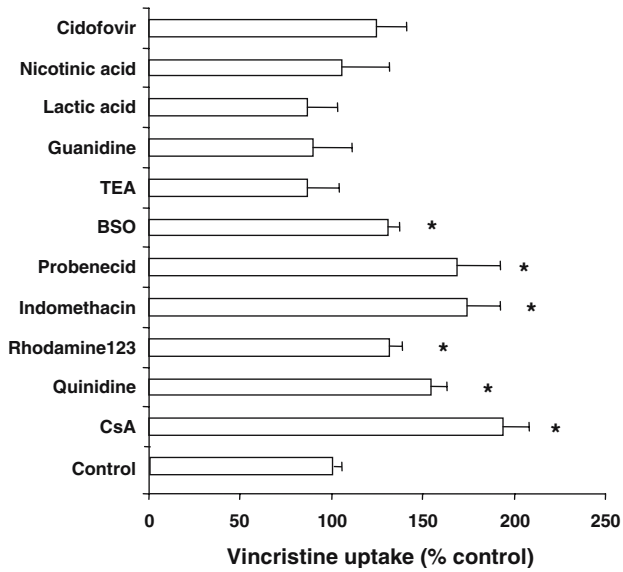

Fig. 7. One-hour uptake of 200 nM vincristine in the presence of P-gp substrates, MRP1 inhibitors, organic cations, or organic anions. Both apical and basolateral compartments of the cell layers were dosed with 200 nM [^{3}H]vincrinstine and one of other agents including a P-gp and MRP1 substrate CsA (10 μM); P-gp substrates: quinidine (100 μM), and rhodamine (100 μM); MRP1 substrates: indomethacin (10 μM) and probenecid (100 μM); organic cations: TEA (100 μM) and guinidine (100 μM); or organic anions: lactic acid (100 μM), nicotinic acid (100 μM), and cidofovir (100 μM). For BSO treatment group, cells were treated with BSO at 100 μM overnight (18 h) before uptake experiment. *Asterisks* indicate a statistically significant difference ($p < 0.05$) from the control.

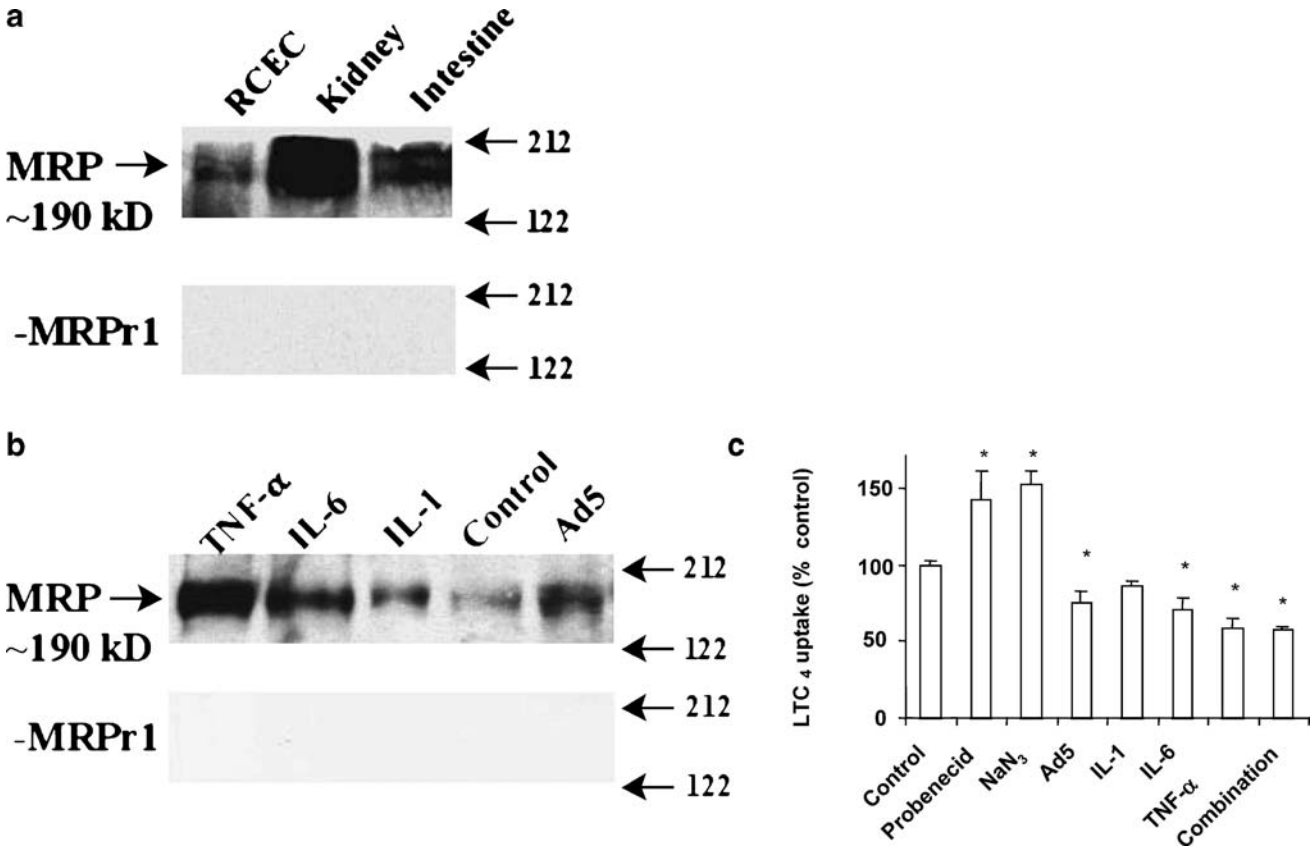

Fig. 8. a. Western blot analysis of MRP1 with MRP1 mAb in membrane proteins of freshly isolated RCEC and positive controls small intestine and kidney. *Upper panel* refers to MRPr1 treatment and *lower panel* negative controls without MRPr1 mAb treatment. **b**. Western blot analysis of MRP1 in membrane proteins of RCEC following various cytokine treatments (IL-1 (10 ng/ml), IL-6 (100 U/ml), and TNF-α (100 U/ml)) or with Ad5 inoculation. **c**. One-hour uptake of LTC_4 (at 10 nm from basolateral compartment) in RCEC in the presence of 100 μM probenecid, or following a pretreatment with 10 mM NaN_3/glucose free medium for 30 min, Ad5-inoculation (MOI of 10), IL-1 (10 ng/ml), IL-6 (100 U/ml), TNF-α (100 U/ml), and a combination of IL-1, IL6, and TNF-α 48 h before uptake experiments. Values are mean ± s.e.m., $n = 3$–4. *Asterisks* indicate a statistically significant difference ($p < 0.05$) from the control.

increased in cultured RCEC with Ad5-infection (189%), treatment of IL-1 (149%), IL-6 (201%), and TNF-α (216%) compared to the control cells (100%) (Fig. 8b).

Modulation of MRP1 Efflux Function

In the presence of 100 μM probenecid, LTC_4 uptake from basolateral dosing of cultured RCEC was increased (144%). By treating the cells with glucose-free BRS containing 10 mM NaN_3, LTC_4 uptake was increased (154%). LTC_4 uptake was decreased by Ad5 infection (76%), by treatment with IL-1 (87%), IL-6 (72%), TNF-α (59%), and a combination of three cytokines (57%) compared to the control (100%) (Fig. 8c).

DISCUSSION

Molecular and Cellular Characterization of MRP1

The expression of *MRP1* gene in the rabbit conjunctiva was first demonstrated by RT-PCR. Rabbit *MRP1* shares high homology to human *MRP1* (Fig. 1). MRP1 appears to primarily localize at the basolateral side of the rabbit conjunctiva epithelium and cultured RCEC (Fig. 2). This basolateral localization of MRP1 was also the case in kidney epithelial cells (9), hepatocytes (22), and Sertoli cells (10). Functionally, MRP1 in kidney and liver probably is responsible for re-absorption of molecules, while MRP1 in testis is to prevent drug entry to damage the germline cells. On the other hand, MRP1 is localized in the apical membrane of brain capillary endothelial cells (12), restricting drug permeability across blood brain barrier (BBB). MRP1 was found in the apical membrane of respiratory epithelial cells, which may attribute to secretion of drugs into the lumen (5).

Efflux Function of MRP1

Transport of fluorescein, a MRP1 substrate (23), across the cultured RCEC layers was asymmetric, favoring the a-to-b transport (Table I), which can be explained by MRP1-mediated efflux toward basolateral direction. Fluorescein uptake was not dependent on dosing side, implying that MRP1 would pump intracellular drugs out of the cells without affecting drug entry. This mechanism is quite different from the "vacuum cleaner" mechanism of P-gp, which directly pumps the substrate out of the cell membrane

before its entry into the cytoplasm (24). MRP1 function can be inhibited by probenecid in a dose dependent manner (Fig. 4). As shown in Fig. 5, MRP1 inhibitors, probenecid and indomethacin (14) increased fluorescein uptake in RCECs. This was comparable to observations in retinal epithelium (25), brain microvessel endothelial cells (23), and rat astrocyte (26). By pretreating the RCECs with glucose-free BRS containing 10 mM NaN_3 to abolish intracellular ATP, fluorescein uptake was increased, suggesting that the decreased MRP1 activity by ATP depletion resulted in increased fluorescein accumulation. Fluorescein uptake was increased by addition of non-steroidal anti-inflammatory (ocular) drugs (NSAID), diclofenac, ofloxacin and flurbiprofen, but not NSAID cromolyn, a steroidal anti-inflammatory drug prednisolone, and an antiviral cidofovir. This suggests that some anion drugs might not be good substrates for MRP1. NSAIDs including indomethacin, sulindac, tolmetin, ofloxacin, and antimicrobial erythromycin, have been reported to interact with MRP1, thereby enhancing chemotherapeutic drug toxicity to human tumor cells (27) and leukemia cells (28).

Cole and Deeley (2) have proposed two transport mechanisms for the MRP1 pump: export of substrates alone, or co-transport of substrates with GSH. To investigate whether fluorescein transport in RCEC is associated with GSH co-transport, the cells were treated with BSO, an irreversible inhibitor of GSH synthesis. GSH in RCEC (8.97 ± 0.06 nmol/million cells which is comparable with that in HepG2, Hela, Caco-2, MDCK, and retinal muller cells (29)) was depleted with BSO treatment (Fig. 6). However, GSH depletion did not affect fluorescein uptake (Table II), suggesting that GSH might not participate in fluorescein efflux by MRP1. The structural requirement for substrates to be transported alone or co-transported with GSH by MRP1 is not clear. MRP1 substrates like GSH conjugates, e.g., LTC_4 and simple organic anion, e.g., calcein (30), appear to be transported alone. In contrast, unmodified vincristine, aflatoxin B1, VP1, either cationic or neutral compounds are co-transported with GSH (2). Our results of GSH depletion counter the argument that oxidative stress affects MRP1-mediated drug transport. A rank order of substrate specificity for MRP1 according to the V_{max}/K_m ratios was reported, i.e. $LTC_4 > LTD_4 >$ S-(2,4-dinitrophenyl)glutathione > 17 β-glucuronosyl estradiol > monoglucuronosyl bilirubin > 3α-sulfatolithocholyl taurine > GSSG (22). Together with our observations, this order suggests that the export of GSH/GSSG might not be a major function of MRP1 in the rabbit conjunctiva.

Since both P-gp and MRP1 are present in the conjunctiva, it is of interest to assess their relative contribution to drug resistance by using vincristine. Vincristine uptake was increased by co-administration of P-gp/MRP1 substrate CsA (194%); P-gp substrates quinidine and rhodamine 123 (132–154%); and MRP1 inhibitors indomethacin and probenecid (169–175%), but not by organic cations or anions (Fig. 7). In primary culture of rat astrocytes, similar enhancement of vincristine accumulation was observed by CsA (140%), verapamil (160%), probenecid (115%), and sulfinpyrazone (180%) (26). Overnight treatment with BSO caused a significant increase in vincristine uptake (131%). This result is comparable with the observation of 9.2-fold increased sensitivity of a MRP1 overexpressing cells to vincristine with BSO treatment (31). Our data suggest that P-gp and MRP1 might play equally important roles in the efflux of vincristine in the conjunctiva.

Regulation of MRP1 by Ad5 Infection and Cytokines

The significant efflux activity of MRP1 and its basolateral localization implies that MRP1's physiological roles would be distinct from P-gp in the conjunctiva. Given that ocular infection and inflammation are common diseases and impaired response to an inflammatory stimulus was observed in MRP1 knockout mice (17), the MRP1-mediated transport of LTC_4 in RCEC (Table I) led us to hypothesize that MRP1 may have a pathophysiological role in facilitating the delivery of LTC_4, an inflammatory factor inducing the migration of neutrophils and macrophages in host defense in the conjunctiva.

Viral infection is reported to increase MRP1 expression and induce cytokine secretion. For example, human T-cell-leukemia-virus-I increased MRP1 expression in adult T-cell leukemia (ATL) cells (32) and intranasal inoculation of Ad5 in mice induced TNF-α, IL-1, and IL-6 secretion (33). We further evaluated the MRP1 expression and function in conjunctiva by ocular Ad5 infection and cytokine challenge. Our Ad5-infected RCEC model, which showed cell swelling and detachment on Day 6 after Ad5 inoculation (34), allowed us to further evaluate MRP1 expression, function, and modulation in a disease state. Western blot analysis showed the MRP1 expression in rabbit conjunctiva and positive controls intestine and kidney. MRP1 expression in RCEC was increased by Ad5 infection, and up-regulated by treatment with TNF-α, IL-1, and IL-6. This implied the involvement of MRP1 in host immune response. The up-regulation of MRP1 by Ad5 infection could be mediated by those cytokines. Elevated secretion of the proinflammatory cytokines TNF-α, IL-6 was also observed in primary culture of human conjunctival epithelial cells in response to stimuli (IL-1β etc) (35). Moreover, cytokine secretion was reported in other epithelium, including nasal epithelium (36), airway epithelium (37), and intestine epithelium (38). Ocular tissues have evolved sophisticated immune responses in pathophysiological conditions. Various cytokines and immune molecules (IL-1α, IFN γ, IL-3, IL-4, IL-5, IL-6, IL-8, RANTES, TNF-α, TGF-β1, HLD-AR, ICAM-1, and GM-CSF) are found expressed in normal conjunctival epithelial cells or up-regulated in diseased states (e.g. keratoconjunctivitis, Sjogren's syndrome patients) (39,40). Cytokine signaling for upregulation of MRP1 expression in RCEC under inflammatory conditions is worthy of further investigation.

To evaluate the hypothesis that MRP1 overexpression in adenovirally-infected and cytokine treated RCEC is associated with enhanced transport of inflammatory factors, we determined LTC_4 uptake under those conditions. As shown in Fig. 8c, increased LTC_4 uptake by MRP1 inhibition by probenecid or intracellular ATP depletion by NaN_3 confirmed the functional MRP1 in Ad5-infected RCEC. Ad5 infection and cytokine treatment resulted in decrease of LTC_4 accumulation in the RCEC, suggesting enhanced MRP1-mediated efflux of inflammatory factors to the underlying tissues during inflammation. MRP1 efflux func-

tion is correlated well with its protein expression. Stein *et al.* (41) also reported that transfected TNF-α stimulated MRP1 expression at the both gene level and protein level in the lung carcinoma cell lines. In addition, adenoviral infection can regulate the expression and function of other transporters (e.g. organic cation transporter), thereby altering drug transport (42).

In summary, we have demonstrated the basolateral localization of MRP1 and its efflux function of pumping certain anion drugs and conjugates out of the rabbit conjunctival epithelial cells. The physiological role of MRP1 might be associated with host immune response by the transport of substrates including the inflammatory factor, LTC_4, to the underlying tissues. Both Ad5 infection and cytokine treatment can up-regulate MRP1 expression and function. Besides P-gp and MRP1, it is of interest to further evaluate other important efflux pumps in the conjunctiva, such as multidrug resistance protein 2 (43) and breast cancer resistance protein (BCRP) (44) which plays an important role in drug disposition. From the drug delivery point of view, the MRP1 function and up-regulation by Ad5-infection shed a light on utilizing MRP1 for improving drug absorption in the ocular inflammation.

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