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Research Paper

Efflux Protein Expression in Human Retinal Pigment Epithelium Cell Lines

Eliisa Mannermaa,[1,5] Kati-Sisko Vellonen,[2,3] Tuomas Ryhänen,[4] Katriina Kokkonen,[1] Veli-Pekka Ranta,[1] Kai Kaarniranta,[4] and Arto Urtti[2]

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Purpose. The objective of this study was to characterize efflux proteins (P-glycoprotein (P-gp), multidrug resistance proteins (MRP1–6) and breast cancer resistance protein (BCRP)) of retinal pigment epithelium (RPE) cell lines.
Methods. Expression of efflux proteins in two secondary (ARPE-19, D407) and two primary (HRPEpiC and bovine) RPE cell lines was measured by quantitative RT-PCR and western blotting. Furthermore, activity of MRP1 and MRP5 of ARPE-19 cell line was assessed with calcein-AM and carboxydichlorofluorescein (CDCF) probes.
Results. Similar efflux protein profile was shared between ARPE-19 and primary RPE cells, whereas D407 cell line was notably different. D407 cells expressed MRP2 and BCRP, which were absent in other cell lines and furthermore higher MRP3 transcript expression was found. MRP1, MRP4 and MRP5 were identified from all human RPE cell lines and MRP6 was not expressed in any cell lines. The pattern of efflux protein expression did not change when ARPE-19 cells were differentiated on filters. The calcein-AM and CDCF efflux tests provided evidence supporting MRP1 and MRP5 activity in ARPE-19 cells.
Conclusions. MRP1, MRP4 and MRP5 are the main efflux transporters in RPE cell lines. There are differences in efflux protein expression between RPE cell lines.

KEY WORDS: blood retinal barrier; cell model; drug transport; efflux proteins; retinal pigment epithelium.

INTRODUCTION

Drug delivery to the retina and vitreous remains a challenge in ophthalmology because the posterior part of the eye is well protected by blood–retinal barrier (BRB). BRB is similar to blood brain barrier (BBB) that protects the brain from harmful and toxic substances, but also restricts drug permeation (1). BRB consists of the endothelium of the retinal vessels (the inner barrier) and the retinal pigment epithelium (RPE, the outer barrier) (2). Based on current information it is not possible to draw conclusions regarding the relative quantitative importance of the inner and outer parts of BRB (3).

RPE protects the neural retina from the systemically circulating xenobiotics. Systemic drug delivery to the posterior eye segment is feasible only for drugs with a wide therapeutic index, like some antibiotics. Systemic adverse effects prevent this approach in most cases. Therefore, retinal diseases are often medicated by invasive intravitreal injections that result in adequate drug concentrations. The recent introduction of antibodies and aptamer to treat the exudative form of age-related macular degeneration has increased the use of intravitreal injections significantly thereby increasing the risk of complications. Periocular and subconjunctival drug administration is an alternative to intravitreal injections (4), but the drug must cross the sclera and RPE to reach the retina and vitreous. The analysis of Pitkänen *et al.* (5) revealed that the RPE is equal (lipophilic small molecules) or more important barrier (hydrophilic or large molecules) than the sclera. Finally, RPE plays a key role in ocular elimination of drugs after intravitreal administration (6). Both physical (tight junctions, cell membranes) and dynamic (e.g. transporters) factors determine the permeability of RPE that has influence on drug delivery, efficacy, and toxicity after intravitreal, subconjunctival, periocular and systemic administration.

As in several other barriers (e.g. blood brain barrier, placenta), the membrane transporters may confer barrier selectivity to the BRB (3). Transporters show usually polarized distribution in the cell membranes and they are classified into influx and efflux transporters depending on the direction of transport. Efflux proteins of ATP-binding cassette (ABC) family pump drugs from the cells to extracellular space (7). P-glycoprotein (P-gp, ABCB1 encoded by MDR1 gene) which was first identified in cancer tissue confers resistance to various drugs including cancer therapeutics, antibiotics, corticoids, and immunosuppressive agents (7,8).

[1] Department of Pharmaceutics, University of Kuopio, Yliopistonranta 1C, 70210, Kuopio, Finland.
[2] Centre for Drug Research, University of Helsinki, Helsinki, Finland.
[3] Division of Biopharmacy and Pharmacokinetics, University of Helsinki, Helsinki, Finland.
[4] Department of Ophthalmology, University of Kuopio, Kuopio, Finland.
[5] To whom correspondence should be addressed. (e-mail: Eliisa.Mannermaa@uku.fi)

Multidrug resistance proteins (MRP1–6, ABCC1–6 and MRP7–9, ABCC10–12) transport mainly anions and conjugated compounds. The efflux transporters have many common substrates, but there are also clear differences in their substrate specificity. For example, MRP4, MRP5 and MRP8 specialize in the transport of cyclic nucleotides and nucleoside monophosphates (9,10). Breast cancer resistance protein (BCRP, ABCG2) substrates include chemotherapeutics and organic anion conjugates (11). The efflux proteins are involved in drug resistance, protection of tissues from toxic compounds, metabolism and signal transduction (12).

Despite their biological importance, only a few efflux proteins have been studied in RPE cells. P-gp expression was shown in human (13,14) and porcine RPE (1), and in D407 and h1RPE cell lines (14,15). MRP1 protein is present in porcine RPE (1), ARPE-19 cell line, and primary human RPE cells (16). MRP5 was detected recently at the mRNA level in the ARPE-19 cells (17) and human RPE, and at protein level in mouse RPE (18). Several efflux transporters (MRP2, MRP3, MRP4, BCRP) have not been studied in RPE.

Cell culture models are widely used in pharmaceutical research to study drug transport across the cellular barrier. The efflux protein profile of the epithelium or cell culture model may have significant impact on drug transport. Surprisingly, the transport and permeability of RPE and RPE cell models have been only rarely investigated (5,19,20). In fact, there is a need for established cell model for outer BRB. Currently, ARPE-19 is the most widely used RPE cell line in retinal biology. On filter ARPE-19 cell line develops some properties of polarized and differentiated RPE cells (21,22). In this study, our main aim was to characterize the expression of the ABC efflux proteins (MDR1, MRP1–6, BCRP) in three human RPE cell lines (ARPE-19, D407, HRPEpiC) and in bovine primary cells (bRPE).

MATERIALS AND METHODS

Cell Culture

ARPE-19 cell line was obtained from American type culture collection (ATCC, Manassas, VA, USA) and HRPEpiC cell line from ScienCell Research Laboratories (San Diego, CA, USA). The D407 cell line is a kind gift to our laboratory from Dr. Richard Hunt (University or South Carolina, Columbia, SC, USA). Bovine RPE cells were extracted from fresh bovine eyes as previously described (23,24). MDCKII cell lines expressing MDR1, MRP1, MRP2, MRP3, MRP5, HEK293 cell line expressing MRP4 (kindly provided by Dr P. Borst from the Netherlands Cancer Institute, Amsterdam) and Caco2 cell line known to express BCRP (ATCC, Manassas, VA, USA) were used as positive controls in Western blot studies.

D407, MDCKII, HEK293 and Caco2 cells were grown using Dulbecco's Modified Eagle Medium (DMEM) and ARPE-19 and bRPE cells using DMEM-F12 (1:1). The growth media were supplemented with 10% or 5% (D407) fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin. In addition, 2 mM L-glutamine was added to ARPE-19 and bRPE cells. Filter culturing of ARPE-19 cells was done as described earlier (21). Briefly, cells were seeded at a density of 1.6×10^5 cells per cm^2 to laminin (mouse laminin, Becton Dickinson labware, Bedford, MA, USA) coated Transwell filters from Costar (Cambridge, MA, USA). The content of growth medium for filter cultured ARPE-19 cells was as mentioned above with the exception that the volume of FBS was lowered to 1%. The progress of differentiation and polarization was followed by measurement of transepithelial resistance (TER) with Endohm chamber (World Precision Instruments, Sarasota, FL, USA). The cultures with TER greater than 85 $\Omega.cm^2$ were used.

Media and FBS were purchased from Gibco (Invitrogen, Carlsbad, CA, USA), L-glutamine and penicillin/streptomycin from EuroClone (Pero, Italy). Cell culture reagents used for HRPEpiC cells were supplied by ScienCell Research Laboratories (San Diego, CA, USA). The cells were cultured at 37°C in 5% or 7% (ARPE-19) CO_2 atmosphere.

Extraction of RNA and Quantitative RT-PCR

In flask cultures the cells were collected for RNA extraction when they reached confluency, in filter cultures the cells were grown for 4 weeks. The total RNA was extracted using TRI-Reagent® (Sigma, St. Louise, MO, USA), treated with DNAse (DNA free, Ambion, Austin, TX, USA), quantified using RiboGreen quantification assay (Molecular Probes, Netherlands) and reverse transcribed (M-MuLV, Fermentas, Hanover, MD, USA).

MDR1, MRP2 and BCRP mRNA levels were measured by quantitative RT-PCR (qRT-PCR) using custom made primers and probes (25). FAM-labeled Assay on Demand TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) were used to measure mRNA levels of MRP1 (Hs00219905_m1), MRP3 (Hs00358656_m1), MRP4 (Hs00195260_m1), MRP5 (Hs00981071_m1) and MRP6 (Hs00184566_m1). The designed probes span an exon–exon junction and thus will not amplify genomic DNA. The PCR-control samples were RNA without M-MuLV enzyme and cDNA reaction without RNA. A total of 40 ng of each cDNA was amplified using ABI Prism 7000 system (Applied Biosystems, Foster City, CA, USA).

The amounts of transcripts were calculated based on DNA standard curves derived from expression plasmids of MDR1 (26), MRP1 (27), MRP2 (28), MRP3 (29), MRP4 (30), MRP5 (31), MRP6 (32) and BCRP (33). Briefly, the region containing the sequence was cut with appropriate restriction enzymes and purified from gel after electrophoresis separation. Concentration of DNA was quantified with PicoGreen reagent (Molecular probes, Netherlands). The standard curves were created each time with the samples.

Western Blotting

Western blotting was used to confirm the expression at the protein level of those efflux proteins that were expressed at the RNA level in RPE cells. The whole cell protein extracts were run on 7% SDS-PAGE gel and wet-blotted onto nitrocellulose membrane (Amersham Biosciences, Pittsburgh, PA, USA) overnight. Ponceau S staining (Sigma, St Louis, MO, USA) was used to ensure equal loading of proteins. The membranes were blocked in 3% fat free dry milk in 0.3% Tween/PBS for 1 h at room temperature. All

primary antibodies were diluted in 0.5% BSA in 0.3% Tween/PBS (MRP1 (MRPr1) 1:5,000, MRP2 (M2 III-6) 1:4,000, MRP4 (M4I-10) 1:5,000, MRP5 (M5I-1) 1:1,000 and BCRP (BXP-21) 1:2,000) and mouse (MRP2 1:8,000, BCRP 1:2,000) and rat (MRP1 and MRP4 1:10 000, MRP5 1:1,000) secondary antibodies in 3% fat free dry milk in 0.3% Tween/PBS. Incubations for primary and secondary antibodies were conducted for 1 h at room temperature. All primary antibodies were from Abcam (Cambridge, UK) and secondary antibodies from Amersham Biosciences (Pittsburgh, PA, USA). Protein–antibody-complexes were detected with the enhanced chemiluminescence method (Millipore, Billerica, MA, USA).

Calcein-AM Assay to Test General Efflux Activity

Efflux protein activity of ARPE-19 and bRPE cells was assessed with the calcein-AM assay. Cells were incubated with calcein acetoxymethyl ester (calcein-AM, Calbiochem, La Jolla, CA, USA), a cell permeable compound that is metabolized by intracellular esterases yielding a fluorescent, cell impermeable calcein. Calcein is a substrate of MRP1 and MRP2 and calcein-AM of P-gp and MRP1 (40,41). The inhibition of efflux proteins causes calcein accumulation. This is quantified as increased intracellular fluorescence. The concentrations (15 μM cyclosporin A (Calbiochem, La Jolla, CA, USA), 200 μM progesterone (Sigma, St Louis, MO, USA), 500 μM verapamil (ICN Biomedicals, Irvine, CA, USA) and 100 μM MK571 (Cayman Chemicals, Ann Arbor, MI, USA)) of each inhibitor were chosen based on the preliminary experiments with wide concentration ranges.

The assay was conducted as described earlier with minor modifications (34). In brief, ARPE-19 cells were cultured on 96-well plates for four days prior to the assay. The cells were pre-incubated for 15 min at 37°C in the HBSS (BioWhittaker, Cambrex, Belgium) buffered with 25 mM Hepes (BioWhittaker, Cambrex, Belgium), pH 7.4 in the presence of inhibitors or in the buffer (control). Calcein-AM was added to yield final concentration of 2 μM and incubation was continued for a further 20 min. The test solutions were replaced with ice-cold buffer and the cellular fluorescence was measured by Victor 1420 Multilabel Counter (Wallac, Finland) using 480 nm for excitation and 535 nm for emission. The results are expressed as the percentage of fluorescence compared to control (100%).

CDCFDA/CDCF Assay to Test MRP5 Activity

Carboxydicholorofluorescein (CDCF) has been used previously as a surrogate marker for MRP5 mediated transport (35). CDCFDA, a diacetate ester of CDCF, enters the cells by passive diffusion, while CDCF is able to cross cell membranes only by active transport mediated by MRP2, MRP3 or MRP5 (31,36,37). Probenecid, a non-specific inhibitor of organic anion transporters, was used as an inhibitor of this system.

ARPE-19 cells were cultured for four days prior to the assay. The assay was performed at 37°C using a horizontal plate mixer (Heidolph inkubator 1000 and Titramax 1000, Heidolph Electro GmbH & Co., Keiheim, Germany). The growth medium was aspirated and the cells were pre-incubated in HBSS, 25 mM Hepes (pH 7.4) for 30 min. The cells were then incubated in the presence of 5 μM CDCFDA for 20 min. Subsequently, the parent compound was aspired, cells were washed twice and the fresh buffer either with or without probenecid (1 mM or 10 mM) was added to the cells. The buffer samples from the wells were collected at 30 and 90 min. Fluorescence of the samples was measured simultaneously with the CDCF standard curve by Victor 1420 Multilabel Counter (Wallac, Finland) using 485 nm for excitation and 535 nm for emission.

Statistical Analyses

All statistical analyses were performed using SigmaStat 3.5 software (SSPS Inc., Chicago, IL, USA). Kruskal–Wallis analysis was used to compare multiple experimental groups. When the difference was significant ($p<0.05$), the comparisons *versus* control group (calcein-AM assay) or pairwise comparisons (CDCFDA/CDCF-assay) were conducted with the Dunn's test.

RESULTS

Quantitative RT-PCR

The expression of eight efflux proteins was studied at the RNA level in human RPE cell lines (Fig. 1). The mRNA expression levels are reported as transcript copy number/40 ng cDNA. This allows direct comparison of expression levels between different efflux proteins. MRP1, MRP4 and MRP5 transcripts were found in all cell lines. MRP2 and BCRP were detected only in the D407 cell line. In addition, MRP3 expression was notably higher in D407 cells compared to the other cell lines. MDR1 was found in the D407 cell line and at a low level in one batch of the HRPEpiC cells. Only trace amounts of MRP6 transcripts were detected in all the studied cell lines.

Expression of efflux proteins between flask (non-differentiated) and filter cultured (differentiated) ARPE-19 cells were compared. Expression levels of MRP1, MRP4 and MRP5 were 17%, 88% and 193% higher in differentiated cells than in non-differentiated cells, respectively. The overall expression profile was not changed (Fig. 2).

Western Blots

Pattern of protein expression was fairly similar to mRNA expression. MRP1, MRP4 and MRP5 were found in human RPE cell lines (Fig. 3). Unlike MRP1 and MRP4, MRP5 was not detected in bRPE cells (Fig. 3). MRP2 and BCRP proteins were detected from the D407 cell line, but not from ARPE-19, HRPEpiC or bRPE cells (Fig. 4). However, it is possible that MRP2, MRP5 and BCRP primary antibodies did not react with the bovine proteins.

Calcein-AM Assay

Similar efflux activity was seen in ARPE-19 and bRPE cells by measuring calcein retention in the presence of efflux inhibitors (Fig. 5). Since neither P-gp nor MRP2 are

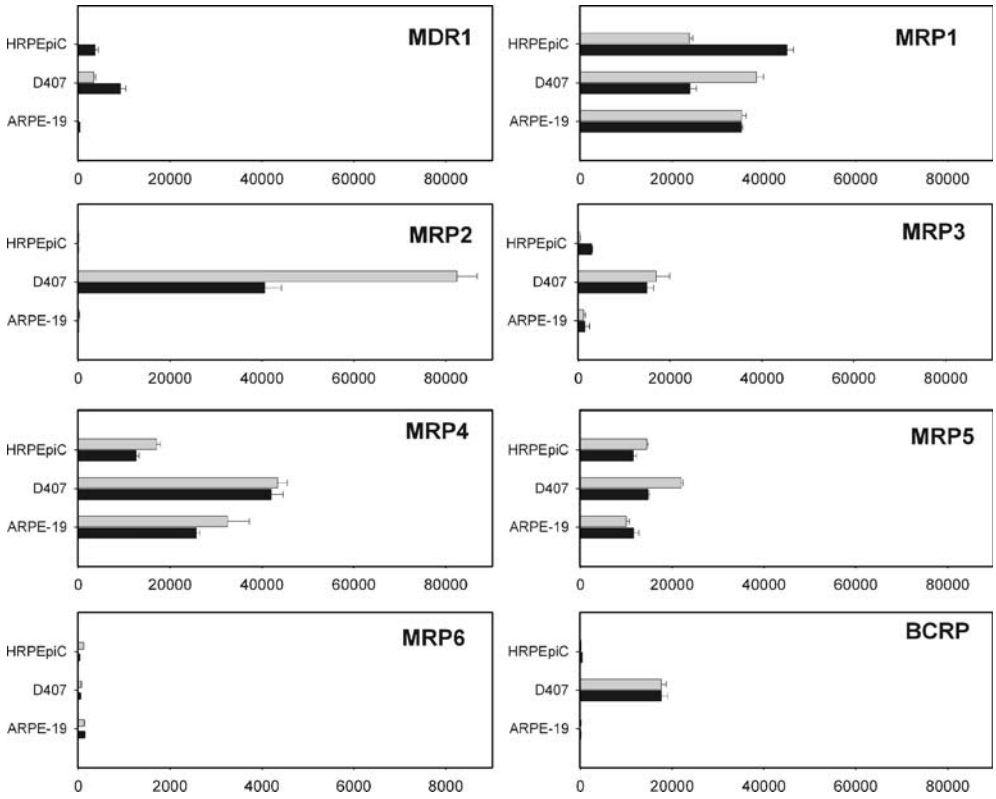

Fig. 1. Messenger RNA expression levels of efflux proteins in human RPE cell lines. Data are expressed as messenger RNA copy number/40 ng cDNA from two independent cell batches. Each measurement had three replicates. The data are expressed as the mean±SD.

expressed in ARPE-19 cells (Figs. 1 and 4), the results point to MRP1 activity in ARPE-19 cell line.

CDCFDA/CDCF Assay

The CDCFDA/CDCF-assay provided evidence of MRP5 activity in ARPE-19 cells. In the presence of probenecid, the effluxed CDCF levels in the buffer were significantly ($p<0.05$) lower compared to the levels without the inhibitor (Fig. 6). An effect of increasing inhibitor concentration could be detected at 90 min, however this was not statistically significant.

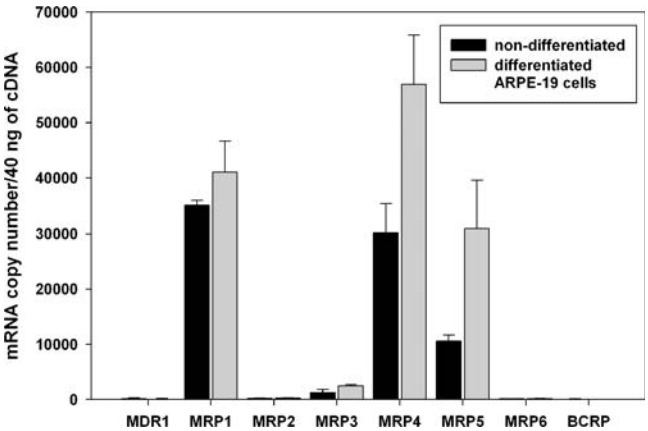

Fig. 2. Messenger RNA expression levels of efflux proteins in non-differentiated and in differentiated ARPE-19 cells. Data are expressed as messenger RNA copy number/40 ng cDNA from three independent cell batches. Each measurement had three replicates. The data are expressed as the mean±SD.

DISCUSSION

The importance of efflux transporters in pharmacokinetics has become evident during the past decade (38). Since efflux transporters of RPE are poorly characterized (3), we studied

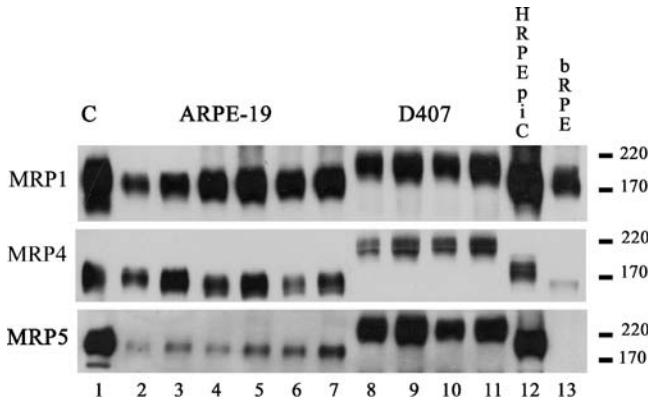

Fig. 3. Expression of MRP1, MRP4 and MRP5 in ARPE-19, D407, HRPEpiC and in bRPE cells by Western blotting. Lanes loaded with different amounts of total protein. *Lane 1* (*C*) positive control: MDCK-MRP1 (15 μg) for MRP1, HEK293-MRP4 (10 μg) for MRP4 and MDCK-MRP5 (2 μg) for MRP5, *lanes 2–7* ARPE 19 cells (*lanes 2*, *4*, *6* 25 μg, *lanes 3*, *5*, *7* 50 μg) from three different cell batches, lanes *8–11* D407 cells (*lanes 8*, *10* 25 μg, *lanes 9*, *11* 50 μg) from two different cell batches, *lane 12* HRPEpiC (50 μg) and *lane 13* bRPE (50 μg).

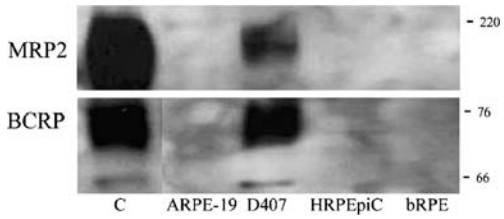

Fig. 4. Expression of MRP2 and BCRP in ARPE-19 (50 μg), D407 (50 μg), HRPEpiC (50 μg) and bRPE cells (35 μg) by Western blot. MDCKII-MRP2 (15 μg) was used as a positive control (*C*) for MRP2 and Caco2 cell line (50 μg) for BCRP.

their expression and activity in RPE cell lines. Efflux proteins can restrict or enhance drug transport across the RPE depending on the direction of drug permeation and location of the efflux transporters in polarized RPE.

Expression of the efflux proteins was different in D407 cells compared to ARPE-19, HRPEpiC and bRPE cells (Figs. 1, 3 and 4). Especially MRP2, MRP3 and BCRP were expressed at high levels in the D407 cell line. Previously Constable *et al.* showed P-gp expression in D407 cells, and lack of expression in ARPE-19 cells, results which are in line with this study (15). MRP proteins of D407 cells also had higher molecular weights compared to the other cells suggesting glycosylation differences (Fig. 3). As a conclusion, there are differences between RPE models.

Expression of MRP4 has not been reported previously in RPE. Expression of MRP5 has been studied only at mRNA level in human material and at protein level in mouse RPE (17,18). We observed MRP4 and MRP5 expression in ARPE-19, D407 and primary human RPE cells at RNA and protein levels (Figs. 1 and 3). In addition MRP4 protein could also be found from bRPE. MRP5 protein was not detected by western blot from bRPE, but this could be also due to fact that the primary antibody did not react with bovine protein.

CDCF is a high affinity substrate of MRP5 (35). CDCF may also be effluxed by MRP2 and MRP3 (31,36,37). The use of the CDCF to study MRP5 efflux activity in ARPE-19 cells was based on its high affinity and on the results from expression studies. MRP2 is not expressed in ARPE-19 cells

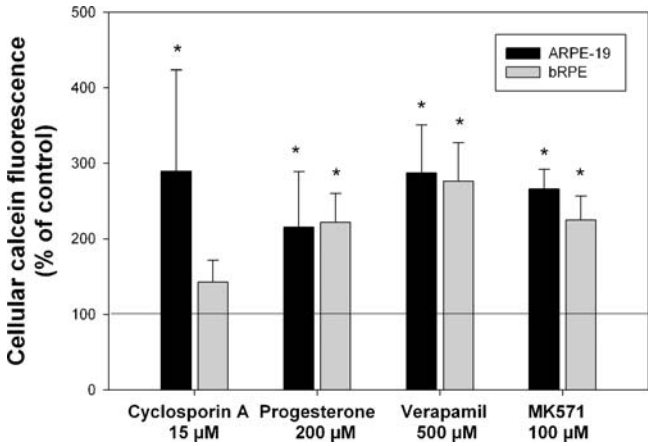

Fig. 5. Calcein retention in ARPE-19 and bRPE cells in the presence of efflux protein inhibitors. The results are expressed as a percentage of fluorescence relative to control (control=100%). The studies were repeated at least three times and each measurement had three replicates. The data are expressed as the mean±SD, $*p<0.05$.

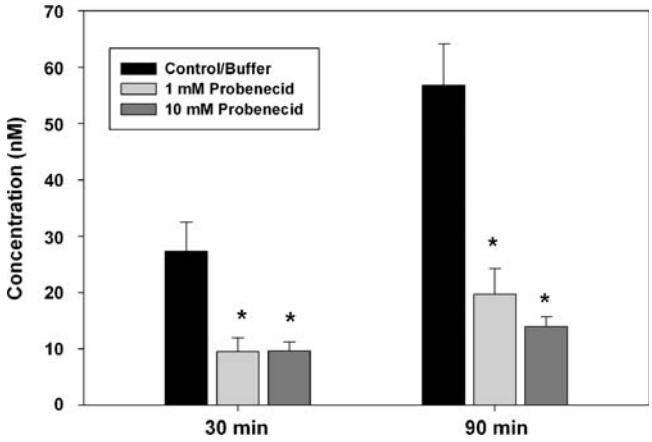

Fig. 6. CDCF efflux from ARPE-19 cells in the absence and in the presence of an inhibitor, 1 mM and 10 mM probenecid. The results are from three independent experiments each having at least four replicates. The data are expressed as the mean±SD, $*p<0.05$.

and MRP3 transcript is expressed only at low level. This is a first study to show MRP5 activity in RPE cells.

It has been suggested that MRP4 and MRP5 may take part in cAMP and cGMP homeostasis and they may affect distribution of nucleoside analogue drugs (10,39). In the eye cAMP and cGMP have several functions as second messengers in phototransduction (40), in the absorption of subretinal fluid (41), and in phagocytosis of ROD outer segments (42,43). Some ocular drugs interact with MRP4 or MRP5 (e.g. atzidothymidine, ganciclovir and 5′-fluorouracil, see ref. 3). Furthermore, vincristine, etoposide and teniposide, retinoblastoma drugs, interact with MRP4 (vincristine) and MRP5 (etoposide and teniposide) (44,45). However the exact role of MRP4 and MRP5 in drug resistance is currently unknown.

The direction of transport of P-gp and MRP1 substrates is towards blood in the BRB (1). MRP1 and P-gp may help to remove metabolic end products and other substrates across the outer BRB to choroidal circulation. Probenecid sensitive active transport of fluorescein and some antibiotics from vitreous to the choroid has been demonstrated, but the responsible transporter has not been identified (46,47). Aukunuru *et al.* showed efflux of fluorescein in ARPE-19 cells (16). We were also able to show further evidence of MRP1 activity in ARPE-19 cells by calcein-AM assay (Fig. 5). In calcein-AM assay the results from ARPE-19 and bRPE cells were similar except when cyclosporin A was used as an inhibitor (Fig. 5). From our experience, also with other cell lines, we think that this could be due to its high lipophilicity and possible non-specific binding to cell culture materials and cells. In addition species differences in efflux protein substrate specificity and binding are possible.

There are differences in efflux protein profiles between inner and outer BRB (1,13–18,48–52, this study). P-glycoprotein and BCRP proteins have been found in inner BRB (48–51) and in a recent study by Tachikawa *et al.* MRP3, MRP4 and MRP6 transcripts were detected in mouse retinal vascular endothelial cell fraction (52). In RPE cells significant levels of MRP3 and BCRP were only expressed in D407 cells and MRP6 could not be detected from any of RPE cell lines. Similar MRP6 results have been found in an earlier study, where MRP6 mRNA was found in retina, but not from RPE

of the patients with pseudoxanthoma elasticum (PXE) (53,54).

It is also possible that the process of cell culture may affect the expression of the efflux proteins. However, culturing ARPE-19 cells on filter did not change the expression profile, but the overall level of efflux protein expression was higher (Fig. 2). Our group has also studied efflux protein expression in corneal epithelium. The expression profile of the efflux proteins in the human corneal epithelial cell lines and corneal epithelium *in vivo* is different from RPE cell lines (Vellonen *et al*. 2009, submitted manuscript) suggesting differences between the efflux barriers in the anterior and posterior eye.

CONCLUSION

We demonstrated here the expression profile of the efflux transporters in different RPE cells. ARPE-19 cell line was found to be similar with primary cells, whereas D407 cell line was notably different. It is clear that of the expression of the efflux transporters in the outer BRB warrants further studies on their role in ocular pharmacokinetics and toxicology.

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