Expression of Efflux Transporters in Human Ocular Tissues

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ABSTRACT

To investigate the expression profiles of efflux transporters in human ocular tissues, quantitative real-time polymerase chain reaction, Western blotting, and immunohistochemistry were used to obtain the relative mRNA and protein expressions of various efflux transporters in human ocular tissues. The cornea, conjunctiva, iris-ciliary body (ICB), retina and choroid, human corneal epithelial cell line (HCEC), and human retinal pigment epithelial cell line (ARPE-19) were examined for the expressions of multidrug resistance–associated proteins 1–7 (MRP1–7), multidrug resistance 1 (MDR1) P-glycoprotein, lung resistance protein (LRP), and breast cancer-resistance protein (BCRP). The expression sites and patterns of efflux transporters were significantly different in ocular tissues, HCEC, and ARPE-19, as well as the expression profiles of efflux transporters in mRNA and protein levels in ocular tissues. At the protein level, MRP1–7, MDR1, and LRP were expressed in the corneal epithelium; MRP1–7, MDR1, LRP, and BCRP were expressed in the conjunctival epithelium; MRP1–2, MRP6–7, MDR1, and LRP were expressed in the ICB; MRP1–3, MRP6–7, MDR1, and LRP were expressed in the retina; MRP1–3, MRP6–7, MDR1, and LRP were expressed in the HCEC; and MRP7, MDR1, LRP, and BCRP were expressed in the ARPE-19. This quantitative and systematic study of efflux transporters in normal ocular tissues and cell lines provides evidence of cross-ocular tissue transporter expression differences, implying that efflux transporter expression variability should be taken into consideration for better understanding of ocular pharmacokinetic and pharmacodynamic data.

Introduction

The eye is a complex organ with sophisticated anatomic and physiologic structures (Sasaki et al., 1999; Edelhauser et al., 2010; Ulbrich and Lamprecht, 2010). These structures include various ocular barriers, such as corneal epithelium, the blood-aqueous barrier (BAB), and the blood-retina barrier (BRB), which govern the entry and exit of nutrients and xenobiotics into and out of ocular tissues in the anterior and posterior parts of the eye, making it a challenge to deliver drugs effectively into ocular tissues (Mannermaa et al., 2006; Zhang et al., 2008; Mitra, 2009). Topically applied drugs may be rapidly eliminated from the precorneal area (Wu et al., 2010). The cornea, a major pathway for eye drops or eye ointments to enter the eye, is also an important barrier to drug penetration (Ghate and Edelhauser, 2006; du Toit et al., 2011; Pahuja et al., 2012; Tartara et al., 2012). Additional factors preventing drugs from reaching the target site of the anterior chamber involve drug elimination through aqueous humor outflow and entering the uveal blood circulation via the BAB (Gaudana et al., 2010). Systemic administration is one possible route for delivery of drugs to the back of the eye; however, retinal transfer of drugs from the circulating blood is strictly regulated by two blood-ocular barrier systems: the BAB and the BRB (Gaudana et al., 2010).

The multidrug resistance (MDR) phenotype, a major type of cell resistance toward xenobiotics, remains the main cause of failure in cancer chemotherapy (Wu et al., 2011). The MDR phenomenon is associated with a decrease in intracellular xenobiotics/drug accumulation by efflux transporters, such as multidrug resistance 1 (MDR1) P-glycoprotein, multidrug resistance–associated proteins (MRPs), breast cancer-resistance protein (BCRP, also known as ABCG2), and lung-resistance protein (LRP). These proteins have been subsequently detected in normal tissues of kidney, intestine, adrenal gland, liver, blood-brain barrier, and cells of the immune system. The presence of these proteins in the apical membranes of normal tissues, such as renal tubular cells and intestinal absorptive cells, can interfere with drug availability, metabolism, and toxicity, because they reduce intestinal absorption and increase renal secretion of a wide range of drugs and metabolites, which are substrates for these membrane transporters (Bodo et al., 2003; Delou et al., 2009).

Many transporters have been discovered in tissues of the liver, kidney, and intestine (Obligacion et al., 2006; Klaassen and Lu, 2008; Delou et al., 2009; Klaassen and Aleksunes, 2010). Among them, efflux transporters and influx transporters are related to drug delivery...
The role of efflux transporters has been investigated more intensively than that of influx transporters, due to the defense mechanism of efflux transporters against penetration of xenobiotics (Eechoute et al., 2011). Prominent efflux transporters identified in tissues belong to the ATP binding cassette superfamily, including MDR1, MRPs, and BCRP (Giacomini et al., 2010). LRP is not an ATP binding cassette transporter but a major vault protein (Bouhamyia et al., 2007). As it is thought to drive drugs away from the nucleus, LRP has also been reported as an efflux transporter (Leonard et al., 2003; Bouhamyia et al., 2007), although there are discrepancies about its MDR functions (Mossink et al., 2002).

Efflux transporters lower bioavailability by effluxing the molecules out of the cell membrane and cytoplasm, and they are important determinants of drug accumulation in target organs or cells and of overall drug uptake, distribution, and excretion. Moreover, some efflux transporters have been discovered in ocular tissues, such as the cornea and retina (Kajikawa et al., 1999; Kennedy and Mangini, 2002; Becker et al., 2007; Vellonen et al., 2010). Similar to the liver, kidney, and intestine tissues, the expression and function of efflux transporters at various layers of ocular epithelial and endothelial cells may significantly influence ocular drug efficacy by means of absorption, distribution, and elimination (Dey et al., 2004; Hariharan et al., 2009).

However, knowledge about these efflux transporters in ocular tissues is very limited, despite the potentially important role they may play in ocular drug disposition. The data of expression and function available only involve lower species and cell lines, and some even remain controversial.

Expression and location profiles are important prefactors to functional protein research (Dallas et al., 2006). With some differences in the results, expression profiles of efflux transporters in human corneas have been evaluated (Becker et al., 2007; Zhang et al., 2008; Mossink et al., 2002).

Fig. 1. mRNA expression levels of efflux transporters in human ocular tissues. The data are expressed as the mean ± S.D. a: $P_{CE-cornea}<0.05$, b: $P_{CE-ICB}<0.05$, c: $P_{CE-RC}<0.05$, d: $P_{conjunctiva-cornea}<0.05$, e: $P_{conjunctiva-ICB}<0.05$, f: $P_{conjunctiva-RC}<0.05$. ($n = 6$ for CE, ICB, and RC; $n = 4$ for conjunctiva). As MRP3 in ICB and RC, and MRP4 in CE had no expression, the statistical analysis was not performed here.
Vollenen et al., 2010). However, there have been few reports on the expression pattern of efflux transporters in the other human ocular drug-absorption barrier tissues, such as conjunctiva, iris-ciliary body (ICB), and retina-choroid (RC). In the present study, we characterized the expression and location profiles of 10 major efflux transporters in ocular tissues. In addition, the expression profiles of human corneal epithelial cells (HCEC) and human retinal pigment epithelial cells (ARPE-19), two cell lines commonly used as in vitro models for corneal and retinal drug absorption studies, respectively, were investigated and compared.

Materials and Methods

**Human Ocular Tissues.** The human ocular tissues from donors with no history of eye disease were provided by the Eye Bank of Shandong, International Federation of Eye and Tissue Banks (Qingdao, China). The eye globes, enucleated within 10 hours of death, were immediately dissected, and the conjunctiva, ICB, and RC were collected. Patient data are summarized in Table 1. Human corneal epithelium (CE) tissues were obtained from patients who underwent photorefractive keratectomy surgery for correction of refractive errors at Qingdao Eye Hospital, Shandong Eye Institute (Qingdao, China). Written informed consent for photorefractive keratectomy and CE to be used in this research was obtained from each patient. Patients with manifested ocular pathology or topical ocular drug therapy were excluded. The CE was scraped off before photoablation. Each tissue was snap-frozen in liquid nitrogen or topical ocular drug therapy were excluded. The CE was obtained from each patient. Patients with manifested ocular pathology or topical ocular drug therapy were excluded. The CE was scraped off before photoablation. Each tissue was snap-frozen in liquid nitrogen and stored at −80°C for protein and RNA analysis. Normal human corneas that were left after corneal transplantation surgery and were not suitable for further clinical application were provided by Qingdao Eye Hospital, as well as the conjunctiva, ICB, and retina used for immunohistochemical analysis. All tissues were from donors free of ocular disease and other systemic complications. This study was approved by the Ethical Review Committee of Shandong Eye Institute, and the handling of donor tissues was consistent with the tenets of the Declaration of Helsinki regarding the protection of donor confidentiality.

**Cell Culture.** Simian virus 40–immortalized HCECs were kindly provided by Prof. Choun-Ki Joo (School of Medicine, the Catholic University of Korea, Seoul, Korea). The cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 (1:1) media, 5% fetal bovine serum (Gibco-BRL, Grand Island, NY), 5 μg/ml insulin (Sigma, St. Louis, MO), 0.1 ng/ml cholela toxin (EMD Biosciences, San Diego, CA), 10 ng/ml human epidermal growth factor (R&D Systems, Minneapolis, MN), and 0.5% dimethyl sulfoxide (Sigma) in a humidified 5% CO2 incubator at 37°C.

**ARPE-19.** (ATCC, catalog no. CRL-2302) cells were cultured in 1:1 of Dulbecco’s modified Eagle’s medium/nutrient mixture F12 (Invitrogen, Carlsbad, CA), containing 10% fetal bovine serum (Invitrogen). The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The culture medium was changed every 3 days.

**Construction of Plasmid and Standard Curves.** The target DNA fragments of corresponding genes obtained by chemical synthesis were ligated into the pMD19-T vector (Takara, Dalian, China) at 16°C overnight. The ligation mixtures were transformed into competent Escherichia coli DH5α cells. After being confirmed by polymerase chain reaction (PCR) and sequencing, the positive clones were designated as recombinant plasmid pMD19-MRP1, pMD19-MRP2, pMD19-MRP3, pMD19-MRP4, pMD19-MRP5, pMD19-MRP6, pMD19-MRP7, pMD19-MDR1, pMD19-LRP, and pMD19-BCRP.

The correct recombinant plasmids were extracted as standard plasmids for fluorescence quantitative polymerase chain reaction (PCR) using a plasmid extraction kit (Tiangen Corp, Beijing, China). A Smartspec3000 spectrophotometer (Bio-Rad Corp, Hercules, CA) was used to determine the concentration of recombinant plasmids by measuring the absorbance at 260 nm, and purity was confirmed using a 260/280 nm ratio. The plasmid copy number was calculated using the following formula: molecules μl−1 = (A260 × 6.022 × 10^15) (660 × B)^−1, where A is the plasmid concentration (g μl−1), B is the plasmid length containing the cloned sequence, 6.022 × 10^23 is the Avogadro’s number, and 660 is the average molecular weight of one base pair.

**Quantitative Detection of Samples.** Quantitative real-time PCR was performed according to previous descriptions with minor modification (Perini et al., 2011). Total RNA was isolated from each tissue according to the manufacturer’s protocol (NucleoSpin RNA II System; Macherey-Nagel, Düren, Germany) and subjected to reverse transcription at 42°C for 60 minutes in a 40-μl reaction mixture using a first-strand cDNA synthesis kit (Takara). The

| TABLE 1 | Patient demographic information |
|---|---|---|---|---|
| Tissues | Donor No. | Age (yrs) | Gender | Eye | Endothelial Cell Density (/mm²) |
| Cornea, Conjunctiva, Iris-Ciliary Body, and Retina-Choroid | 1 | 18 | Male | OD | 2197 |
| | 2 | 38 | Male | OD | 2341 |
| | 3 | 15 | Female | OD | 2550 |
| | 4 | 29 | Female | OD | 2470 |
| | 5 | 29 | Female | OD | 3250 |
| | 6 | 45 | Female | OD | 3180 |
| Cornea Epithelium | 1 | 15 | Male | OD | 2790 |
| | 2 | 19 | Male | OD | 2570 |
| | 3 | 27 | Male | OD | 2830 |
| | 4 | 16 | Female | OD | 2590 |
| | 5 | 18 | Female | OD | 2830 |
| | 6 | 25 | Female | OD | 2830 |

Each dilution was tested in triplicate and used as an amplification template to construct standard curves by plotting the plasmid copy number logarithm against the Ct values under optimum conditions. Sequence detection system software (7500 System; Applied Biosystems, Singapore) was used to create the standard curves and calculate the correlation coefficients. Positive control (standard plasmid without dilution) and negative control experiments were conducted in parallel for quality control.
reagents (Tiangen) and sequence detection system were used in fluorescence quantitative PCR as recommended by the manufacturer. The primers and oligonucleotide probes used are listed in Table 2. Cycling conditions were as follows: 10 minutes at 95°C, followed by 40 cycles of amplification for 15 seconds at 95°C and 1 minute at 60°C. The expected fragment length was 150 bp. Quantification data were analyzed by the Sequence Detection System software. The log-linear portion of the fluorescence versus cycle plot was extended to determine a fractional cycle number at which a threshold fluorescence was obtained (threshold cycle), and this number was used as a reference for each analyzed gene. The cDNA samples were amplified in parallel with plasmid standards in each run, and their Ct values were plotted together with the standard curves, from which the target gene mRNA copy numbers were determined.

**Western Blotting and Antibodies.** Western blotting was performed as previously described (Chen et al., 2012), and the antibody information is listed in Table 3. The total protein was prepared from each tissue using radioimmunoprecipitation assay buffer (50 mmol/l Tris, 150 mmol/l NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, sodium orthovanadate, and sodium fluoride, pH 7.4; Galen, Beijing, China) at a density of approximately 4.0 × 10⁴ cells/35-mm dish in complete medium and grown to subconfluence. The medium was removed, and the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 minutes at 25°C, and three washes with 10 ml TBST were performed between each step. The membranes were then developed with SuperSignal West Femto Maximum Sensitivity substrate (Pierce Biotechnology, Rockford, IL) and exposed to X-ray film (Kodak, Rochester, NY). The immunoreactive bands were quantified using National Institutes of Health Image 1.62 Software (National Institutes of Health, Bethesda, MD). All the experiments in this study were performed three times, and the results were reproducible. For each sample, the levels of proteins of interest were normalized to that of GAPDH. Primary antibodies included antibodies against MRP1, MRP2, MRP3, MRP4, MRP6, MRP7, BCRP, MDR1, and LRP (Santa Cruz Biotechnology, Santa Cruz, CA); anti-MRP5 antibodies (Abcam, Cambridge, MA); and anti-GAPDH antibody (Kangchen, Shanghai, China).

**Immunohistochemistry and Immunocytochemical Staining.** Each tissue was fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical analysis. Paraffin sections, 4 μm in thickness, were deparaffinized, rehydrated, and stained with antibodies, using routine protocols. The antibody information is shown in Table 3. 3,3′-Diaminobenzidine and 3-amin-9-ethylcarbazole, originally introduced for the localization of horseradish peroxidase, have been widely used for staining in immunohistochemistry. The cornea and conjunctiva were stained with 3,3′-diaminobenzidine, which can form a brown precipitate upon oxidation. The cornea and conjunctiva were stained with 3-amin-9-ethylcarbazole, with which a red precipitate can form when exposed to oxidation. The cornea and conjunctiva were stained with 3,3′-diaminobenzidine, which can form a brown precipitate upon oxidation. The cornea and conjunctiva were stained with 3-amin-9-ethylcarbazole, which can form a red precipitate upon oxidation. The cornea and conjunctiva were stained with 3,3′-diaminobenzidine, which can form a brown precipitate upon oxidation. The cornea and conjunctiva were stained with 3-amin-9-ethylcarbazole, which can form a red precipitate upon oxidation.

Cells were plated in a glass culture dish (Biosung Biotech Co., Wuxi, China) at a density of approximately 4.0 × 10⁴ cells/35-mm dish in complete medium and grown to subconfluence. The medium was removed, and the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 minutes at 25°C.
minutes, followed by three phosphate-buffered saline washes. The fixed cells were incubated with 0.2% Triton-100 in phosphate-buffered saline for 5 minutes, blocked in 5% bovine serum albumin, and incubated with primary antibodies for 30 minutes, followed by incubation with corresponding fluorescence-conjugated secondary antibodies for 1 hour. Images were obtained using an Eclipse TE2000-U confocal laser-scanning microscope (Nikon, Tokyo, Japan).

Data Analysis. Data are presented as mean ± S.D. Difference comparison of measurement data among groups was determined with single factor variance analysis, and comparisons between two groups were performed with the least significant difference t test (Fig. 1). The differences among groups were analyzed using Student’s t test (Fig. 2). SPSS 11.5 software (SPSS Inc., Chicago, IL) was used, and P < 0.05 was considered significant.

Results

mRNA Expression in Human Ocular Tissues, HCEC, and ARPE-19. The rank order of MRP1 expression was RC > ICB > conjunctiva; MRP2 was RC > ICB > conjunctiva > CE; MRP3 was conjunctiva > CE, and it was absent in RC and ICB; MRP4 was ICB > conjunctiva > RC, and it was absent in CE; MRP5 was RC > ICB > CE > conjunctiva; MRP7 was RC > CE > conjunctiva > ICB; MDR1 and BCRP were ICB > RC > conjunctiva > CE; and LRP was CE > ICB > conjunctiva > RC. MRP6 was absent in all the ocular tissues (Fig. 1).

The mRNA expression of MDR1 in HCEC was absent, while that of other efflux transporters in the HCEC was significantly higher than the CE; even MRP4 had a high expression, but it was absent in the CE. However, in ARPE-19, the mRNA expression profile was somewhat sophisticated—MDR1 was absent in ARPE-19; significantly higher expression than the RC in MRP1, MRP4, and LRP; and significantly lower in MRP2, MRP5, MRP7, and BCRP. As in the ocular tissues, MRP6 was absent from the HCEC and ARPE-19 (Fig. 2).

Western Blotting Studies of Protein Expression in Human Ocular Tissues. Western blotting analysis of protein expression in human ocular tissues is presented in Fig. 3. The quantitative comparison of the fold difference in the expression of efflux transporters is shown in

![Fig. 2. mRNA expression levels of efflux transporters in HCEC, ARPE-19, CE, and RC. Data are expressed as mean ± S.D. (n = 6 for CE and RC; n = 3 for HCEC and ARPE-19). *P < 0.05. As MRP3 in RC, MRP4 in CE, and MDR1 in HCEC and ARPE-19 had no expression, the statistical analysis was not performed here.](image-url)
Fig. 4. MRP1, MRP3, MRP6, MRP7, and LRP were found in the CE, while only faint MDR1 expression was observed in the CE. MRP1, MRP3, MRP4, MRP6, MRP7, MDR1, BCRP, and LRP were detected in the conjunctiva. MRP1-7, MDR1, and LRP were found in ICB. MRP1, MRP2, MRP3, MRP5, MRP6, MRP7, MDR1, BCRP, and LRP were detected in the RC. MRP1, MRP2, MRP3, MRP7, MDR, BCRP, and LRP were observed in the HCEC. Expression of MRP7, MDR1, BCRP, and LRP was evident in ARPE-19.

**Localization of Efflux Transporters in Human Ocular Tissues.**

The expression sites of efflux transporters differed among the ocular tissues. In the human cornea, MRP1, MRP2, and MRP6 were found to be predominant in the basal cell layer of the corneal epithelium, as well as faint MRP3 and MRP4; MRP7 and MDR1 were detected in the whole corneal epithelium, with a mild expression of LRP (Fig. 5).

In the human conjunctiva, the MRP1, MRP7, and LRP expressions were found in the whole conjunctival epithelium; the MRP2, MRP3,
MRP4, MRP6, MDR1, and BCRP expressions were detected in the basal cell layer. The MRP5 expression was detected in the upper layer of the conjunctival epithelium (Fig. 6).

In the human ICB, expressions of MRP1, MRP2, and MDR1 were observed in the stromal cells (Fig. 7). High MRP1, MRP2, MRP6, and MDR1 expressions, as well as faint MRP3, MRP7, and LRP expressions were detected in the whole retina (Fig. 8). The immunohistochemical localization of drug transporters in human ocular tissues is summarized in Table 4.

Localization of Efflux Transporters in the HCEC and ARPE-19.

The results of immunofluorescence microscopy are summarized in Figs. 9 and 10. There were also great differences in the expression sites of efflux transporters between the HCEC and ARPE-19. In the HCEC, MRP1, MRP2, MRP3, MRP6, MRP7, MDR1, and LRP were observed in the whole cytoplasm, while MRP4, MRP5, and BCRP were absent. In ARPE-19, MRP7, MDR1, LRP, and BCRP were detected in the whole cytoplasm, while MRP1-6 was absent (Table 4).

Discussion

Transporter functions may reflect factors of location (within a tissue, as well as within cells), the level of expression, substrate and inhibitor specificity, and functional kinetics (Dallas et al., 2006). It is important to formulate a clear description of the expression profiles of the efflux transporters in ocular tissues. In this study, several barriers in ocular drug absorption, through topical or systemic administration, were chosen to elucidate their efflux transporter expression profiles in mRNA and protein levels. A summary of this study’s results, as well as some expression reports of efflux transporters in human ocular tissues, is shown in Table 5.

The Cornea and HCEC.

The corneal epithelium is lipoidal in nature and contains 90% of the total cells in the cornea; it displays significant resistance toward permeation of topically administered hydrophilic drugs. Kawazu et al., (1999) first reported the presence of MDR1 in cultured rabbit corneal epithelial cells, where it could help protect cells from being damaged by uptake of substances (Kawazu et al., 1999). In 2003, Dey et al. (2003) found, for the first time, that the human cornea can express MDR1. Since then, individual efflux transporters in human and animal corneas and corneal epithelial cells have been widely investigated (Karla et al., 2007a,b, 2009; Pelis et al., 2009; Vellonen et al., 2010; Barot et al., 2011; Li et al., 2012). Functional activity of some efflux transporters has also been evaluated, but the results of efflux transporter expressions and functions were very different. Dey et al. (2003) and Becker et al. (2007) investigated the mRNA expression of MDR1 in human corneas, as well as the MDR1 expression in a human corneal cell line by Western blotting, but no detectable or very low MDR1 mRNA and/or protein expressions were observed in human corneas according to other reports (Dey et al., 2003;
Becker et al., 2007), and these discrepancies may be due to variation in sensitivity of different experimental methods and different tissues used (Vellonen et al., 2010). Regarding the function of efflux transporters, Dey et al. (2003) evaluated the functional activity of MDR1 efflux pump with cultured rabbit primary corneal epithelial cells and a corneal cell line (Statens Seruminstitut rabbit cornea [SIRC] cells) as the model, with the results that MDR1 affected corneal uptake. Becker et al. (2007) evaluated the potential value of different epithelial cell culture systems as in vitro models for investigation of corneal permeability. Transformed human corneal epithelial cells, SIRC cells, SkinEthic human corneal epithelium, Clonetics human corneal epithelium, excised rabbit corneas, and human corneas were involved; however, MDR1 and similar efflux systems were observed to have no significant effects on corneal permeability. Discrepancies have been found not only in the expression of efflux transporters at the mRNA and protein levels, but also in different corneal models; expressions may even fluctuate in the same cell models, depending on the maturation status of cells (Vellonen et al., 2006; Verstraelen, 2011; Juuti-Uusitalo et al., 2012).

There have been many reports on the expression and function of efflux transporters in animal corneal cell models; however, to our knowledge, only a few of them are related to the efflux transporter expression in human corneas, and the results are conflicting. In the current study, MDR1 displayed 9.11 ± 2.33 Qty/ng RNA expression in the CE, similar to the results of other reports (Becker et al., 2007; Vellonen et al., 2010). MRP1, MRP2, MRP3, MRP7, and BCRP also had low mRNA expressions, while MRP5 and LRP had relatively high mRNA expressions. However, discrepancies in the expression of efflux transporters at the mRNA and protein levels were also found in our results, such as MRP5 and MRP6. BCRP, as a stem cell marker, which was reported to express in human corneas (Saghizadeh et al., 2011), showed
Fig. 7. Representative figures of immunolocalization of efflux transporters in human iris-ciliary body. Arrow indicates the localization of efflux transporters. The red color was seen in positive cells. Nuclei were counterstained in blue with hematoxylin.
Fig. 8. Representative figures of immunolocalization of efflux transporters in human retina. Arrow indicates the localization of efflux transporters. The red color was seen in positive cells. Nuclei were counterstained in blue with hematoxylin.
very faint mRNA and failed to be detected by Western blotting in this study. The reason may be that stem cells in the cornea amounted to <1% of total cells, and BCRP existed in the limbal epithelium but not in the stem cell–free corneal epithelium (Budak et al., 2005).

Polarization and stratification seem to have only slight effects on the efflux protein expression in HCE cells, since only small differences were detected between the HCE model and nonconfluent HCE cells (Vellonen et al., 2010), so only nondifferentiated state of the HCEC

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<th>Ocular Tissue</th>
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<th>MRP5</th>
<th>MRP6</th>
<th>MRP7</th>
<th>MDR1</th>
<th>LRP</th>
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<td>Corneal Epithelium</td>
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<td>Conjunctiva Epithelium</td>
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<td>Iris-Ciliary Body</td>
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<td>Retina</td>
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<td>ARPE-19</td>
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was used in the current study (The ARPE-19 was performed like this). Our results showed that cell lines or primary cells differed substantially from freshly isolated human corneas or corneal epithelium in terms of efflux transporter expressions, as previously reported (Becker et al., 2007; Xiang et al., 2009; Vellonen et al., 2010).

**Conjunctiva.** The conjunctival layer plays a role in the drug absorption of timolol maleate and carbonic anhydrase inhibitors to the posterior segment of the eye, and the conjunctival epithelium is the main barrier (Urtti, 2006; Gaudana et al., 2010). It was reported that MDR1 existed in rabbit conjunctival epithelial cells and impaired the

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**Fig. 10.** Representative figures of immunolabeling of efflux transporters in ARPE-19. Arrow indicates the expression of efflux transporters.
absorption of the immunosuppression drugs such as cyclosporine A and β-blocker propranolol (Saha et al., 1998; Yang et al., 2000). Therefore, it is important to understand the expression and regulation of efflux transporters, as well as their effects on drug transport in the conjunctiva. Our study seems to be the first report on efflux transporter expression profiles in the human conjunctiva. In mRNA expressions, all of the efflux transporters except MRP6 were observed; at the protein level, MRP1, MRP3, MRP4, MRP6, MRP7, MDR1, BCRP, and LRP could be detected in the conjunctiva.

Iris-Ciliary Body. The blood-aqueous barrier resides in the iris and ciliary body epithelium. In the iris, the capillary endothelium is not fenestrated, but there are tight junctions. In the ciliary body, there are tight junctions. The nonpigmented epithelium of the ciliary body represents an important component of the BAB of the eye. Many therapeutic drugs penetrate poorly across the nonpigmented epithelium into the aqueous humor of the eye interior (Pelis et al., 2009). Previously, only individual efflux transporters in the ICB were investigated. Zhang et al. (2008) observed MRP1, MRP3, and BCRP mRNA expressions in the ICB, finding that MRP2 had an extremely low expression; MDR1, MRP3, and BCRP had a low expression; MRP1 had a medium expression in this tissue. Dey et al. (2003) confirmed the BCRP expression in the ciliary epithelium and iris pigment epithelium in adult human eyes by RT-PCR and immunofluorescence studies. In this study, we first reported efflux transporter expression profiles in the human ICB. Only MRP3 and MRP6 could not be detected in mRNA expressions; MRP5 and BCRP could not be detected at the protein level. The results of immunohistochemistry also showed that MRP1, MRP2, and MDR1 had high expressions in the stromal cells of the ciliary body.

Retina-Choroid and ARPE-19. The retina is composed of neural retina and retinal pigment epithelium cells, which are adjacent to the choroid (Zhang et al., 2008). The BRB resides in the retina and consists of two major components: the endothelium of retinal blood vessels (inner BRB) and the retinal pigment epithelium (outer BRB). Although the inner and outer BRBs together create a “privileged” environment in the retina and vitreous, the outer BRB constitutes the major absorption barrier for transcellularly or topically administrated drugs. The retina-choroid is commonly used as a tissue-level model to study the permeability of the outer BRB. As an ex vivo model, the retina-choroid can also be a useful tool for identification and characterization of carrier-mediated systems on

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<th>Ocular Tissue</th>
<th>mRNA RFR</th>
<th>MRP1</th>
<th>MRP2</th>
<th>MRP3</th>
<th>MRP4</th>
<th>MRP5</th>
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<th>MRP7</th>
<th>MDR1</th>
<th>LRP</th>
<th>BCRP</th>
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<td>NR</td>
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<td>NR</td>
</tr>
</tbody>
</table>

RFR, results from reports; RFTM, results from this manuscript; IH, immunohistochemistry; IF, immunofluorescence; NR, no report.

Velflenon et al., 2010.
Zhang et al., 2008.
Becket et al., 2007.
Dahlin et al., 2013.
Dey et al., 2003.

TABLE 5
Summary of expression reports of efflux transporters in human ocular tissues

<table>
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<tr>
<th>Ocular Tissue</th>
<th>mRNA RFR</th>
<th>MRP1</th>
<th>MRP2</th>
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<th>MRP4</th>
<th>MRP5</th>
<th>MRP6</th>
<th>MRP7</th>
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<tr>
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Int J Pharm, finding that MRP2 had an extremely low expression; MDR1, MRP1-3, and BCRP mRNA expressions in the individual efflux transporters in the ICB were investigated. Zhang et al. (2008) observed MRP1, MRP3, and BCRP mRNA expressions in the ICB, finding that MRP2 had an extremely low expression; MDR1, MRP3, and BCRP had a low expression; MRP1 had a medium expression in this tissue. Dey et al. (2003) confirmed the BCRP expression in the ciliary epithelium and iris pigment epithelium in adult human eyes by RT-PCR and immunofluorescence studies. In this study, we first reported efflux transporter expression profiles in the human ICB. Only MRP3 and MRP6 could not be detected in mRNA expressions; MRP5 and BCRP could not be detected at the protein level. The results of immunohistochemistry also showed that MRP1, MRP2, and MDR1 had high expressions in the stromal cells of the ciliary body.
Efflux Transporters in Human Ocular Tissues

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the RPE (Kansara and Mitra, 2006). ARPE-19, a commercially available human RPE cell line, is usually selected as a model of RPE in studies of transport, protein expression/secretion, and barrier breakdown (Zhang et al., 2006; Nevala et al., 2008). Therefore, in this study, the RC and ARPE-19 were used to determine and compare the difference in the efflux transporter expression. The retinal and choroidal tissue complex is composed of multiple layers, and the RPE is just one of the cell types in the RC. To our knowledge, there have been only two reports on efflux transporter mRNA expressions in the human retina (Zhang et al., 2008; Dahlin et al., 2013). Zhang et al. investigated MDR1, MRP1-3, and BCRP mRNA expressions and found that all these efflux transporters had mRNA expressions (Zhang et al., 2008). But the protein expression profiles in the human retina were not concerned. In the current study, no mRNA expression of MR3 and MR6, and relatively low expressions of MR4 and MR7 were detected, but others had high expressions. At the protein level, we only failed to observe MR4, and MR1, MR2, MR6, and MDR1 were further verified as having relatively high expressions in the retina.

When compared with the human retina-choroid, ARPE-19 had a significantly different efflux transporter expression profile. Therefore, ARPE-19 should be used with caution when in vitro models are used in the efflux transporter research (Constable et al., 2004; Mannermaa et al., 2009).

Many ocular tissues exist that are not major barriers to ocular drug delivery but express various efflux transporters (Merriman-Smith et al., 2002; Miyazawa et al., 2007). Further investigations may be needed.

Conclusion

This article showed the mRNA and protein expression profiles of efflux transporters in human ocular tissues. The wide differences in expression among tissues and cell lines indicate that investigations on efflux transporter functional activity should be based on their natural expressions in ocular tissues, and cell lines should be used with caution when in vitro models are involved.

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Authorship Contributions

Participated in research design: Wu, P. Chen.
Conducted experiments: P. Chen, H. Chen, Jiang, Han.
Contributed new reagents or analytic tools: Zhang, M. Chen.
Performed data analysis: Wu, P. Chen.
Wrote or contributed to the writing of the manuscript: Wu, P. Chen.

References

Barot M, Gokulchandri MR, Haghnejadgah M, Dulvi P, and Mitra AK (2011) Effect of emergence delivery but express various efflux transporters (Merriman-Smith et al., 2002; Miyazawa et al., 2007). Further investigations may be needed.


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