mRNA Expression of Metabolic Enzymes in Human Cornea, Corneal Cell Lines, and Hemicornea Constructs

Christian Kölln and Stephan Reichl

Abstract

Purpose: Drugs from ophthalmic formulations are mainly absorbed into the eye via the corneal route. However, little is known about drug metabolism during the transcorneal passage. The objective of this study was to determine the mRNA expression of phase I and II isoenzymes in human corneal epithelial tissue, corneal cell lines, and a tissue-engineered cornea equivalent (a hemicornea construct) as *in vitro* model for drug absorption studies. **Methods:** The reverse transcription polymerase chain reaction was used to profile the mRNA expression of 10 cytochrome P450 enzymes (CYP) and seven phase II enzymes in the three human corneal cell lines and the hemicornea construct. The human corneal epithelial cell line (HCE-T), human corneal keratocyte cell line (HCK-Ca) and human corneal endothelial cell line (HENC) were used. Human liver tissue, human corneal epithelium from donor corneas, and the human colon adenocarcinoma cell line Caco-2 were also investigated. **Results:** All the phase I and II mRNAs were expressed in the human liver tissue. The Caco-2 cell line showed an expression pattern similar to the liver tissue, although the signals for CYP1A2 and CYP3A4 were absent. In the case of the donor human corneal epithelium, all the detected phase I mRNAs had lower levels than did the liver tissue. By contrast, the phase II mRNA expression pattern was heterogeneous to the liver tissue. The expression patterns in the three human corneal cell lines were comparable, although the signals for a few phase I enzymes and N-acetyltransferase (NAT2) mRNAs were only detectable in the HCE-T. In the hemicornea construct, all the investigated phase I and II mRNA (except for CYP1A2, CYP2B6, CYP2C19, and NAT2) were expressed. **Conclusions:** Overall, the mRNA expressions of the tested phase I and phase II enzymes in the hemicornea construct and the three corneal cell lines correlated well with the expression patterns of the *ex vivo* human corneal epithelium.

Introduction

EYE DISEASES ARE TREATED with topically administered drugs in most cases, and the cornea represents the main absorption route for the majority of these substances.^{1–3} Due to the tight junctions of the epithelial layer, efflux transporter, tear flow, and small surface of the eye, however, drug bioavailability is typically between 5% and <1%.^{3–5} Human metabolic pathways can be divided into phase I and phase II reactions. The purpose of the phase II reactions is to transform xenobiotics in more water-soluble compounds via conjugation and to facilitate their elimination through the renal pathway. By contrast, phase I reactions often support conjugation reactions by either unmasking or introducing functional groups through oxidative, reductive, or hydrolytic reactions. Additionally, phase I and phase II reactions are capable of detoxifying xenobiotics.

In the eye, metabolic activity is responsible for inactivating ophthalmic drugs and activating prodrugs, such as latanoprost or valacyclovir.^{6,7} During drug development, transcorneal permeation studies are performed to detect the possible influences of the metabolic system on the active substances. Due to the poor availability of human donor corneas, animal test models are normally used for these studies. Because of the various disadvantages of animal experiments (i.e., the need to sacrifice animals for experimental purposes, the need for high numbers of expensive laboratory animals, and the individual differences between animals), however, a standardized *in vitro* model of the human cornea is required. Various corneal cell culture models have already been developed,^{8–17} and most have been well characterized for permeation experiments.^{6,16–22} With the exceptions of the Clonetics® model (cHCE) introduced by Lonza⁵ and of esterase activity in the human corneal construct,²³ metabolic

activity has not yet been comprehensively characterized in corneal cell culture models.

Therefore, the aim of the present study was to determine the mRNA expression of phase I and II isoenzymes in human corneal epithelial tissue, corneal cell lines, and a human hemicornea construct in the context of *in vitro* models for drug absorption studies. Cytochrome P450 (CYP) enzymes are one of the most important phase I enzymatic families. Therefore, the CYP isoenzymes CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 were investigated. In the case of the phase II enzymes, representatives of the important enzymatic families were chosen. Isoenzymes of glutathione transferase (GSTA1-1, GSTO1-1, GSTP1-1), N-acetyltransferase (NAT1 and NAT2), a sulfotransferase isoenzyme (SULT1A1), and UDP-glucuronosyltransferase UGT1A1 isoenzyme were examined.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, Eagle's minimal essential medium (MEM), fetal calf serum (FCS), insulin, epithelial growth factor (EGF), and nonessential amino acids (NEAA) were obtained from Biochrom (Berlin, Germany). Keratinocyte basal medium (KBM) and the KGM[®] SingleQuots Kit CC-4131 were purchased from Lonza (Cologne, Germany). An antibiotic-antimycotic solution (consisting of penicillin, streptomycin, and amphotericin B) and a MycoTrace Mycoplasma PCR Detection Kit were obtained from PAA Laboratories (Pasching, Austria). Dimethyl sulfoxide (DMSO) and ethidium bromide were purchased from Sigma (Deisenhofen, Germany). Tissue culture flasks were obtained from Sarstedt (Nümbrecht, Germany), and Transwell[®] cell culture inserts were purchased from Costar (Farnwald, Germany). TRIzol[®] and all the designed primer pairs were obtained from Invitrogen (Karlsruhe, Germany). The RevertAid[™] First Strand cDNA Synthesis Kit, DreamTaq[™] DNA Polymerase and Random Hexamer Primer were purchased from Fermentas (St. Leon-Rot, Germany).

Human tissue

Specimens of human corneal epithelium pooled from 16 human donors between 18 and 102 years of age were stored in a 0.9% sodium chloride solution at -80°C until processing. A human liver preparation from a 91-year-old donor was stored at the same temperature and was free of known tissue-specific diseases. The human tissues were obtained from the Cornea Bank of Hannover Medical School (Hannover, Germany) in accordance with ethical regulations and processed in accordance with the guidelines outlined in the Declaration of Helsinki.

Cell culture

The human corneal epithelial cell line (HCE-T)²⁴, human corneal keratocyte cell line (HCK-Ca),¹⁴ and human corneal endothelial cell line (HENC)²⁵ were cultivated submerged, which resulted in a confluent monolayer after 7 days. The three SV40-immortalized cell lines were cultivated in a 1:1 mixture of DMEM and Ham's F12 containing 5% FCS, 5 $\mu\text{g}/\text{mL}$

insulin, 10 ng/mL EGF, 0.5% DMSO, and 1% of an antibiotic-antimycotic solution. The colon adenocarcinoma cell line, Caco-2, was cultivated submerged in Eagle's MEM with 20% FCS, 1% NEAA, and 1% antibiotic-antimycotic solution. All the cell lines were cultivated in 25 cm² tissue culture flasks under standard conditions at 37°C in a humidified atmosphere containing 5% CO₂. The cells were passaged every 7 days, and the growth media was replaced three times per week. The absence of myoplasmic contamination was determined using the MycoTrace Mycoplasma PCR Detection Kit.

The hemicornea construct was cultivated as described elsewhere.²⁶ Briefly, the hemicornea construct was created step-by-step in Transwell cell culture inserts using a keratocyte growth medium (KGM) containing KBM and the KGM SingleQuots CC-4131 kit, which consisted of bovine pituitary extract, human epithelial growth factor, insulin, hydrocortisone, gentamicin, and amphotericin B. A type I collagen gel containing $8 \cdot 10^5$ stromal keratocytes (HCK-Ca) was cast on a permeable polycarbonate filter (1.1 cm² surface area; 3.0 μm pore size). Subsequently, $1 \cdot 10^6$ epithelial cells (HCE-T) were seeded on the collagen lattice and grown while submerged for 6 days, until confluence. After 4 days, the HCE-T cells were lifted to the air-liquid interface to induce multilayered cell growth. The growth media was replaced at days 3, 6, 7, 8, and 9.

Reverse transcription polymerase chain reaction

The reverse transcription polymerase chain reaction (RT-PCR) was used to profile the expression of the 17 phase I and II mRNAs. The isolation of the total RNA from the specimens and cell lines was performed using TRIzol according to the manufacturer's protocol. Homogenization with an MM301 Ball Mill (Retsch, Haan, Germany) at 30 Hz for 10 min using glass beads with diameters of ~ 0.50 to 0.75 mm was performed before the RNA isolation. The purity and concentration of the extracted RNA was spectrophotometrically determined, based on the absorbance at 260 and 280 nm using a Spekol 1300 UV spectrometer (Analytik Jena, Jena, Germany). To convert the total RNA to cDNA, the RevertAid First Strand cDNA Synthesis Kit and Random Hexamer Primer were used according to the manufacturer's protocol. The reverse transcription was performed with a labcycler thermocycler (SensoQuest, Göttingen, Germany) using 5 μg of total RNA.

Primer pairs (see Table 1) specific for the phase I and II mRNA were custom-designed using the nucleotide database of the National Center for Biotechnology Information (NCBI). Subsequently, the specificity of the nucleotide sequences was confirmed by the Primer Basic Local Alignment Search Tool (Primer BLAST) of the NCBI. A three-step PCR protocol using the human liver tissue or Caco-2 cell line as a positive control (depending on the references) was used to detect the optimal annealing temperature of each primer pair.^{27–39} The PCR was run using a 12-step temperature gradient with an interval of 2.2°C between each step. This protocol was run at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, then by the different temperature steps of the gradient for 30 s and finally by 72°C for 1 min. The PCR was stopped after a cycle at 72°C for 5 min. A three-step PCR protocol using DreamTaq DNA Polymerase was performed after the cDNA synthesis. The PCR was run at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, by a primer-

TABLE 1. THE SEQUENCES OF FORWARD (F) AND REVERSE (R) PRIMERS AND CHARACTERISTICS OF THE DESIGNED PRIMER PAIRS

		Primer sequence	Product size [bp]	Annealing temperature T_A [°C]
Phase I	CYP1A2	F: GCC TGG CCC AGA ATG CCC TC R: CCA GGG GGT TCC CGG AGG AG	297	68.9
	CYP2A6	F: TCC CCA GCA CTT CCT GAA TGA R: AGT CTT AGC TGC GCC CCT CT	340	59.2
	CYP2B6	F: AGC TTC GGA AAT CCA AGG GGG C R: AGC TCA AAC AGC TGG CCG AAT	190	62.5
	CYP2C8	F: GTG CAG GAG AAG GAC TTG CCC G R: CAG ATC GGC AGC CAG ATG GGC	200	66.5
	CYP2C9	F: CGG ATT TGT GTG GGA GAA GCC C R: GCG GCA CAG AGG CAA ATC CAT	145	69.1
	CYP2C19	F: CCA CAT GCC CTA CAC AGA TG R: GGT CCT TTG GGT CAA TCA GA	371	60.4
	CYP2D6	F: GGA GCC CAT TTG GTA GTG AG R: TGT TCT GGA AGT CCA CAT GC	186	61.7
	CYP2E1	F: CCA TGC GCA CAG GGA CAG GG R: GGT GGG GTC GAA AGG CTG GC	190	68.7
	CYP3A4	F: AAA GCT CCA TGC ACA TAG CC R: CCA TCA TAA AAG CCC CAC AC	315	64.1
	CYP3A5	F: CCC GAC GTG ATC AGA ACA GTG C R: CTC TGC TTC CCG CCT CAA GTT T	237	69.1
Phase II	GSTA4-4	F: AGA TGG GTT TTA GCT GCC GCC G R: TGG GCA CTT GTT GGA ACA GCA GG	112	69.1
	GSTO1-1	F: GGG AAG AAG CTG TTG CCG GAT G R: AGC AGG GCT GAG ACT GTG GGA T	353	69.1
	GSTP1-1	F: GGA GAC CTC ACC CTG TAC CA R: GAC AGC AGG GTC TCA AAA GG	230	61.4
	NAT1	F: GTC GAT GCT GGG TTT GGA CG R: CCC ACC AAA CAG TGA ACC CCA	347	68.3
	NAT2	F: ATG CTG GGT CTG GAA GCT CCT C R: TGC TCT CTC CTG ATT TGG TCC AGG	139	55.2
	SULT1A1	F: TCT GAA AGA CAC ACC GGC CC R: GGA TCC GTA GGA CAC TTC TCC G	229	64.7
	UGT1A1	F: CAT GCA CTG CCA TGC AGC CT R: GGC AAG GGT TGC ATA CGG GGA A	186	69.1

specific annealing temperature for 30 s and finally by 72°C for 1 min. The PCR reaction was terminated by heating at 72°C for 5 min, followed by rapid chilling to 4°C. The PCR products were separated by 2% agarose gel electrophoresis (PerfectBlue Gelsystem Mini S, Peqlab, Erlangen, Germany) and visualized by ethidium bromide staining using UV light (AlphaImager 1220, Alpha Innotech Corporation, San Leandro, CA).

Results

The functionality of the primer pairs was tested using the human liver tissue^{27–34} and Caco-2 cell line^{35–39} as positive controls, depending on the references. The mRNA expression results for all the enzymes under investigation are shown in Table 2.

Phase I enzymes

All the phase I mRNAs were highly expressed in the human liver tissue. The Caco-2 cell line, by contrast, showed strong mRNA expression of CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A5 mRNA and weak CYP2A6 and CYP2E1 signals. CYP1A2 and CYP3A4 mRNAs were

not detectable in the Caco-2 cells. All the phase I mRNAs were detected at lower levels in the human corneal epithelium than in the liver tissue. The cytochrome P450 isoenzymes CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 showed weak signals, and CYP1A2 and CYP2B6 were absent.

The HCE-T human corneal epithelial cell line showed a phase I mRNA expression pattern comparable to that of the excised human corneal epithelium, except that high levels of CYP2D6 were detected. Both the HCK-Ca and HENC corneal cell lines showed strong CYP2D6 mRNA expression and weak CYP2C8, CYP2C19, CYP2E1, and CYP3A4 mRNA expression. Signals indicating CYP1A2, CYP2A6, CYP2B6, CYP2C9 and CYP3A5 mRNA expression were not detectable.

The CYP2A6, CYP2C8, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 mRNA expression was found to be weak in the hemicornea construct, while CYP2C9 was strongly expressed. CYP1A2, CYP2B6, and CYP2C19 mRNA signals were absent. With regard to the enzyme mRNA expression in the liver tissue and Caco-2 cell line, the hemicornea construct showed the highest expression of CYP2C9 mRNA.

TABLE 2. AN OVERVIEW OF STRONG EXPRESSION (+), WEAK EXPRESSION (◇), OR ABSENCE (–) OF mRNA EXPRESSION FOR PHASE I AND II ENZYMES IN HUMAN LIVER TISSUE, CACO-2, HUMAN CORNEAL EPITHELIUM, IMMORTALIZED HCE-T, HCK-CA, AND HENC CELL LINES AND A HEMICORNEA CONSTRUCT

		Tissue, construct or cell line						
		Human liver	Caco-2	Human corneal epithelium	HCE-T	HCK-Ca	HENC	Hemicornea construct
Phase I	CYP1A2	+	–	–	–	–	–	–
	CYP2A6	+	◇	◇	◇	–	–	◇
	CYP2B6	+	+	–	–	–	–	–
	CYP2C8	+	+	◇	◇	◇	◇	◇
	CYP2C9	+	+	◇	◇	–	–	+
	CYP2C19	+	+	◇	◇	◇	◇	–
	CYP2D6	+	+	◇	+	+	+	◇
	CYP2E1	+	◇	◇	◇	◇	◇	◇
	CYP3A4	+	–	◇	◇	◇	◇	◇
	CYP3A5	+	+	◇	◇	–	–	◇
Phase II	GSTA4-4	◇	+	◇	◇	◇	◇	◇
	GSTO1-1	+	+	◇	+	+	+	+
	GSTP1-1	◇	+	+	+	+	+	+
	NAT1	◇	+	◇	+	+	+	+
	NAT2	◇	◇	–	◇	–	–	–
	SULT1A1	◇	◇	◇	+	+	◇	+
	UGT1A1	+	+	◇	◇	◇	+	◇

HCE-T, human corneal epithelial cell line; HCK-Ca, human corneal keratocyte cell line; HENC, human corneal endothelial cell line.

Phase II enzymes

Every investigated phase II enzyme was found expressed in the liver tissue. Strong GSTO1-1 and UGT1A1 mRNA signals were detected, while GSTA4-4, GSTP1-1, NAT1, NAT2, and SULT1A1 showed weak expression. Compared with the liver tissue, the Caco-2 cell line showed similar or stronger expression of the investigated phase II enzymes. GSTO1-1, NAT2, SULT1A1, and UGT1A1 were detected at the same level, while GSTA4-4, GSTP1-1, and NAT1 had stronger mRNA signals. GSTP1-1 mRNA was strongly expressed in the human corneal epithelium, while GSTA4-4, GSTO1-1, NAT1, SULT1A1, and UGT1A1 had weak expression levels. NAT2 signals were absent.

The GSTA4-4, GSTO1-1, GSTP1-1, and NAT1 mRNA expression patterns were similar in the HCE-T, HCK-Ca, and HENC human corneal cell lines. GSTA4-4 showed weak mRNA expression, while GSTO1-1, GSTP1-1, and NAT1 were strongly expressed. SULT1A1 mRNA was strongly expressed in the HCE-T and HCK-Ca cell lines, and the HENC signals were weak. UGT1A1 mRNA was strongly expressed in the HENC cells but weakly expressed in the HCE-T and HCK-Ca cells. NAT2 expression was absent in the HCK-Ca and HENC cells but was detectable at low levels in the HCE-T cells.

GSTA4-4 and UGT1A1 mRNA expression was found to be weak in the hemicornea construct, while GSTO1-1, GSTP1-1, NAT1, and SULT1A1 were strongly expressed. NAT2 signals were absent.

Discussion

This study investigated the mRNA expression of 17 major metabolic enzymes in the human corneal epithelium, a hemicornea construct, and human corneal cell lines by

RT-PCR. Knowledge of the expression of metabolic enzymes in the affected tissues is important when designing compounds to treat ocular diseases. Prodrugs such as latanoprost and valaciclovir are activated by corneal esterases,^{6,7} while timolol (a nonselective β -adrenergic antagonist used to treat glaucoma) is mainly metabolized in the liver by CYP2D6 (with less than 10% being metabolized by CYP2C19).⁴⁰ Cornea metabolic enzymes may influence timolol; however, the effect does not seem to have clinical significance.⁴¹ Nevertheless, characterizing the expression patterns of metabolic enzymes in the human cornea and in the corneal equivalents used as *in vitro* models for drug absorption studies is essential.

In the present study, a human liver tissue sample^{27–34} and the Caco-2 cell line^{35–39} were used to validate the primer pair functionality and the success of the PCR reaction, depending on the references. mRNAs of all the phase I and II enzymes were detected in the liver tissue. All the cytochrome P450 enzymes and GSTO1-1 and UGT1A1 mRNA were detected at strong levels, while the other phase II enzymes showed less expression. It is notable that in general, all the corneal cell lines, the human corneal epithelium and the hemicornea construct showed lower phase I mRNA expression levels than did the liver tissue, while most of the phase II mRNA had higher or comparable levels. Contradictory data regarding the mRNA expression of CYP2B6,^{36,42} CYP2D6,^{35,36} CYP3A4,^{35,36} and UGT1A1^{43,44} in the Caco-2 cell line have been published in previous studies. In the present study, strong CYP2B6, CYP2D6, and UGT1A1 mRNA expression was detected, while CYP3A4 mRNA was absent. These conflicting results may have been due to the interlaboratory variability of this cell line, as described by Hayeshi et al.³⁵ Therefore, only the human liver tissue was used as a positive control for CYP2B6, CYP2D6, CYP3A4, and UGT1A1 mRNA detection. Specimens of human corneal epithelium, pooled

from 16 donors between 18 and 102 years of age, showed a phase I mRNA expression pattern similar to that documented for the human cornea.²⁸ With the exception of NAT2, mRNA for all the phase II isoenzymes was detected in this tissue. To the best of our knowledge, this is the first report dealing with the phase II mRNA expression of GSTA4-4, GSTO1-1, GSTP1-1, NAT1, NAT2, and SULT1A1 in human corneal tissue.

In the HCE-T cell line, the expression patterns of all the phase I and II mRNAs were found to be similar to those found in the human corneal epithelium. It is notable that the mRNA expression in the HCE-T cell line was either equal to or greater than the expression in the native corneal epithelial tissue we examined. Additionally, NAT2 mRNAs were absent in the human corneal epithelium but detectable in the HCE-T cells at a very low level. These small differences in the HCE-T cells may have been due to the missing influence of the other corneal cell layers, as was the case for the donor epithelium. Comparing the mRNA signals of the HCE-T cells to those of previously established corneal epithelial cell lines, the expression pattern in these cell lines was found to be similar and heterogeneous to the HCE-T cells. The commercially available human corneal epithelial cell line cHCE (Clonetics, Lonza)⁶ showed a phase I mRNA expression pattern comparable to that in the HCE-T cells. However, CYP1A2, CYP2B6, and CYP2E1 mRNA expression was not detected in the cHCE cells. CYP2A6 and CYP2C19 mRNA expression was not detected in the CEPI-17-CL4 human corneal epithelial cell line (which was established by Offord et al. in 1999⁴⁵), although they have been found at high levels in the human cornea.²⁸ The other phase I enzymes investigated in the present study showed similar signals⁴⁵ in the HCE-T cells. Further, CYP3A4 mRNA was not investigated in the CEPI-17-CL4 cells. In contrast to the cHCE cells, data on the phase II mRNA expression in CEPI-17-CL4 cells were available. The GSTP1-1 mRNA expression of the CEPI cell line has also been described as being similar to our results for the HCE-T cells.⁴⁵

Both the HCK-Ca and HENC showed phase I and II mRNA expression patterns similar to those of the HCE-T cells, while signals for CYP2A6, CYP2C9, CYP3A5, and NAT2 were not detectable. These findings indicate a wider range of phase I and II enzyme expression in the HCE-T human corneal epithelial cell line than in cell lines of other corneal cell types (keratocytes, endothelial cells), which is consistent with the theory of Attar et al.⁴⁶ Attar et al. postulated that the highest levels of detoxifying enzymes are located in the corneal epithelium, the outermost layer of the cornea, to protect the eye from external influences.

In the hemicornea construct, the phase I mRNA expression, normalized to the levels found in the human liver, was similar to that described for the human cornea.²⁸ CYP2C19 signals were absent, however. In the monolayers of HCE-T and HCK-Ca cells, which are components of the hemicornea construct, the mRNA expression results for all the phase II enzymes (except for NAT2) and all the cytochrome P450 enzymes (except for CYP2C19) were similar to those of the construct. Due to the much greater number of epithelial cells than stromal cells in the hemicornea construct, the mRNA expression pattern of the corneal equivalent was dominated by the HCE-T cells. The small

differences from the HCE-T cells were likely induced by coculturing with the keratocytes and the more organotypic substrate provided by the collagenous stroma-matrix of the corneal construct.

Overall, the mRNA expression of metabolic enzymes in the corneal cell lines and the hemicornea construct correlated well with the expression pattern found in the human corneal epithelium *ex vivo*. When interpreting these findings, it is important to remember that the mRNA expression does not always correlate with the expression of functional enzymes.⁴⁷ Therefore, detecting phase I and II mRNA only gives a hint of the presence of these enzymes. Additional studies will examine these metabolic enzymes at the protein and functionality level to gain deeper insight into the metabolic pathways of the human cornea and to characterize the hemicornea construct as an *in vitro* model for drug absorption studies.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Stephan Reichl
Institut für Pharmazeutische Technologie
Technische Universität Braunschweig
Mendelssohnstr. 1
Braunschweig 38106
Germany

E-mail: s.reichl@tu-bs.de