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ELISA TOROPAINEN

Corneal Epithelial Cell Culture Model for Pharmaceutical Studies

Doctoral dissertation

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Series Editor: Docent Pekka Jarho, Ph.D.
Department of Pharmaceutical Chemistry

Author's address: Department of Pharmaceutics
University of Kuopio
P.O. Box 1627
FI-70211 KUOPIO
FINLAND
Tel. +358 17 162 405
Fax +358 17 162 252
E-mail: elisa.toropainen@uku.fi

Supervisors: Professor Arto Urtti, Ph.D.
Drug Discovery and Development Technology Center
University of Helsinki

Pekka Suhonen, Ph.D.
Orion Corporation Orion Pharma

Docent Raija Tammi, M.D., Ph.D.
Department of Anatomy
University of Kuopio

Reviewers: Professor Hanna Tähti, Emerita, Ph.D.
Medical School
University of Tampere

Docent Juha Holopainen, M.D., Ph.D.
Department of Ophthalmology
University of Helsinki

Opponent: Professor Jouni Hirvonen, Ph.D.
Division of Pharmaceutical Technology
University of Helsinki

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ABSTRACT

In general, drug absorption into the eye from eye drops is limited. Only 1-7% of the dose eventually reaches aqueous humor since corneal epithelium effectively limits drug delivery into the eye. Gene therapy offers new therapeutic possibilities in ophthalmology, but delivery is an important issue in this case. Ocular drug delivery experiments require sacrification of at least five animals at each time point in the drug concentration profile. Improved corneal cell culture model would therefore be useful in ocular drug and gene delivery experiments, and might reduce the need for animal experiments.

The aim of the study was to develop a cell culture model of corneal epithelium for pharmaceutical studies. The cell culture model was tested as a tool in drug and gene delivery experiments.

Immortalised human corneal epithelial cells (HCE) were grown on collagen or laminin covered permeable support filters with or without feeder fibroblasts. After air-lift the cells stratify and differentiate forming epithelium approximately seven cell layers thick with flattened superficial cells, tight junctions and microvilli. In the optimised cell model the penetration of β-blockers increased with lipophilicity following an almost similar sigmoidal relationship with that of excised rabbit cornea. Paracellular permeability in the HCE-model was generally found to be slightly higher than in the excised rabbit cornea. The HCE-model has larger paracellular pores at lower density than the excised cornea, but overall paracellular space was fairly similar. The HCE-model has esterase activity.

Rabbit corneal epithelium *in vivo* was transfected using non-viral liposomes (1,2-dioleoyl-3-trimethylammonium-propane; DOTAP and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine DOPE) to secrete transgene product (SEAP; a secreted form of human placental alkaline phosphatase) into the tear fluid and aqueous humor. Furthermore, suitability of the differentiated corneal epithelial cell culture for transfection studies was evaluated with DOTAP/DOPE and cationic polymer (polyethyleneimine; PEI). The transfection levels decreased with the increased differentiation of HCE cells. PEI was particularly effective in transfecting the dividing cells but ineffective in the differentiated cells. DOTAP/DOPE also showed high activity in differentiated cell cultures. Significant SEAP expression was seen for at least three days after *in vivo* transfection in the tear fluid and aqueous humor. Rates of SEAP secretion to the basolateral side of differentiated HCE-cells and into the aqueous humor *in vivo* were in the same range showing the predictive applicability of the cell model.

In conclusion, the morphology, physical barrier and permeability properties demonstrate that the HCE-model closely resembles those of the excised rabbit cornea. Corneal epithelium can be transfected topically to reach prolonged protein secretion into the tear fluid and aqueous humor, and the levels of this protein secretion can be predicted correctly with the cell culture model.

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Medical Subject Headings: Eye Diseases/drug therapy; Drug Administration Routes; Drug Delivery Systems; Gene Transfer Techniques; Transfection; Liposomes; Ophthalmic Solutions; Cornea; Epithelium, Corneal; Cells, Cultured; Permeability; Esterases; Alkaline Phosphatase; Rabbits

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Kuopio, April 2007

Elisa Toropainen

ABBREVIATIONS

AAV	adeno-associated virus
ALDH	aldehyde dehydrogenase
BAI1-ECR	brain-specific angiogenesis inhibitor 1
bFGF	basic fibroblast growth factor
β-gal	β-galactosidase
β-gluc	β-glucuronidase
BPE	bovine hypothalamic extract
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
6-CF	6-carboxyfluorescein
CMV	cytomegalovirus
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dnG1	dominant negative mutant cyclin G1
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
FL	fluorescence
GFP	green fluorescent protein
HA	hemagglutin
HCC	human corneal construct
HCE	human corneal epithelial cells
HO-1	heme oxygenase-1
HPLC	high performance liquid chromatography
HPV	human papilloma virus
HStk	herpes simplex virus tymidine kinase

HSV	herpes simplex virus
IL	interleukin
kDa	kilodalton
luc	luciferase
mRNA	messenger ribonucleic acid
MW	molecular weight
OGFr	opioid growth factor receptor
P_{app}	apparent permeability coefficient
P_{cell}	permeability coefficient of cells without filter
PCR	polymerase chain reaction
pDNA	plasmid DNA
PEG	polyethylene glycol
PEI	polyethyleneimine
P_{filter}	permeability coefficient of filter without cells
P-gp	P-glycoprotein
pI	isoelectric point
pKa	dissociation constant
PS	protamine sulfate
RSV	Rous sarcoma virus
RT-PCR	reverse transcription polymerase chain reaction
SEAP	a secreted form of human placental alkaline phosphatase
SV 40	simian virus 40
TEM	transmission electron microscopy
TER	transepithelial electrical resistance
TGF-α	transforming growth factor-α
tPA	tissue plasminogen activator
UV	ultraviolet
VEGF	vascular endothelial growth factor
ZO	zonula occludens
Å	10^{-10} m

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred in the text by Roman numerals **I-III**. Some unpublished data is also included.

I Toropainen E, Ranta V-P, Talvitie A, Suhonen P and Urtti A: Culture model of human corneal epithelium for prediction of ocular drug absorption. Investigative Ophthalmology & Visual Science 42: 2942-2948, 2001

II Toropainen E, Ranta V-P, Vellonen K-S, Palmgrén J, Talvitie A, Laavola M, Suhonen P, Hämäläinen KM, Auriola S and Urtti A: Paracellular and passive transcellular permeability in immortalized human corneal epithelial cell culture model. European Journal of Pharmaceutical Sciences 20: 99-106, 2003

III Toropainen E, Hornof M, Kaarniranta K, Johansson P, Urtti A: Corneal epithelium as a platform for secretion of transgene products after transfection with liposomal gene eyedrops. The Journal of Gene Medicine 9: 208-216, 2007

CONTENTS

1 INTRODUCTION

The eyes provide the majority of information of the outside world to humans, and therefore ocular disorders easily disturb daily life. The eye is effectively protected against foreign substances by its structural features, which is extremely important in the case of microbes and for the maintenance of vital ocular functions. However, these barriers also complicate medical treatment by preventing drug delivery into the eye.

Drug delivery into the eye using topical eye drops is an easy and patient friendly way to treat ocular disorders. Unfortunately, most of the drug is rapidly eliminated from the precorneal area, and eventually only a small amount (1-7%) of the instilled dose actually penetrates the cornea and reaches the aqueous humor (Ghate and Edelhauser 2006). Despite the poor bioavailability the cornea is considered to be a major pathway for ocular penetration of topically applied drugs (Doane et al., 1978). Drug permeability across the ocular surface is highly dependent on the features of the drug molecule; the drug should be neither extremely hydrophilic nor lipophilic. Thus small lipophilic drugs are absorbed into the eye via the cornea whereas large hydrophilic molecules absorb into the eye through the conjunctiva and sclera (Ahmed and Patton 1985; Fig. 1, p. 14). Systemic administration of drugs is not effective due to the blood-aqueous and the blood-retinal barriers (Duvvuri et al., 2003; Ghate and Edelhauser 2006). Furthermore, the use of high doses of administrated drugs to compensate a poor bioavailability may cause systemic and local adverse effects. Ophthalmic drug delivery can also be achieved by periocular and intraocular injections, but these methods are painful, inconvenient and can cause complications in the eye (Sasaki et al., 1999; Sunkara and Kompella 2003). Accordingly, fast degradation of protein drugs effectively prevents their delivery into target cells regardless of the administration method used.

Among other developed drug delivery systems (Ghate and Edelhauser 2006), gene therapy offers new possibilities to overcome the aforementioned problems in corneal drug delivery. In gene therapy a functional gene is inserted into the cell to produce viable proteins using recombinant viruses as a vehicle for gene transfer or techniques that are based on non-viral methods. Cure can be permanent or transient owing to requirement therapy and the expression of the desired transgene can be also targeted, and regulated.

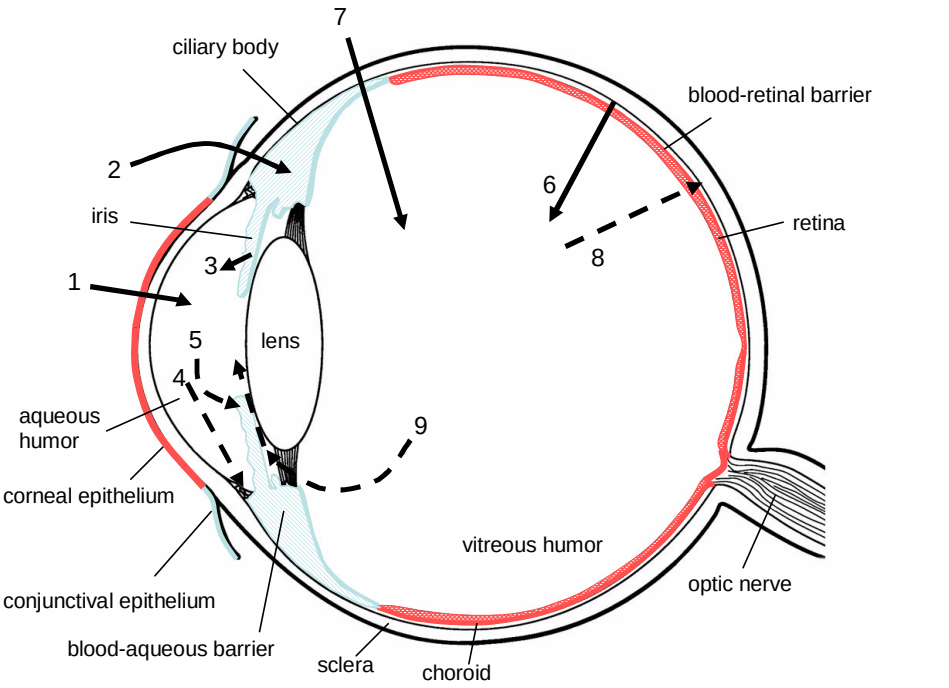

Figure 1. The tight barriers and the pathways of drugs in the eye. The eye structures where the tight barriers locate are indicated in red. The absorption routes are indicated with unbroken and the elimination routes with broken arrows. The main pathway for drugs to enter the anterior chamber is via the cornea (1). Some large and hydrophilic drugs are preferably absorbed via the conjunctival and scleral route, and then diffuse into the ciliary body (2). After systemic administration small compounds can diffuse from the iris blood vessels into the anterior chamber (3). From the anterior chamber the drugs are removed either by aqueous humor outflow (4) or by venous blood flow after diffusing across the iris surface (5). After systemic administration drugs must pass across the retinal pigment epithelium or the retinal capillary endothelium to reach the retina and vitreous humor (6). Alternatively drugs can be administered by intravitreal injection (7). Drugs are eliminated from the vitreous via the blood-retinal barrier (8) or via diffusion into the anterior chamber (9). Reprinted from Hornof et al., 2005 (European Journal of Pharmaceutics and Biopharmaceutics, copyright 2005) with permission from Elsevier Ltd.

Experiments of ocular drug delivery systems are usually performed *in vitro* using isolated rabbit ocular tissues (Chung et al., 1998; Gukasyan et al., 2003; Okabe et al., 2005). Ocular pharmacokinetic studies usually require at least five animals at each time point in every studied drug concentration which means the sacrifice of over 100 rabbits in a typical comparison study of three different drugs or formulations. This kind of animal use is problematic and undesirable for ethical and economical reasons. In

addition, corneal tissue is viable only a few hours after dissection and differences between species may make predictions to human ocular absorption more difficult. Therefore, new methods are needed for ocular pharmaceutical studies.

In principle, the corneal epithelial cell culture models may be useful in drug transport studies (Kawazu et al., 1998, 1999 a and b; Chang et al., 2000). However, these models were based on primary rabbit corneal epithelial cells. They grow only for a few passages and new rabbit cells must be isolated frequently from animals. Therefore, the primary cell models are not useful for large scale screening of new drugs, excipients or delivery systems. Immortalised continuously growing human corneal epithelial cell lines have been mainly used previously for toxicity studies (Kahn et al., 1993; Kruszewski 1997; Ward et al., 1997; Offord et al., 1999). Such cells would be ideal for ocular drug delivery studies, but the barrier formation and permeability features of these models are unknown or poorly studied.

2 REVIEW OF LITERATURE

2.1 Anatomy of cornea

The cornea is a clear, avascular tissue, which protects the anterior parts of the eye from external injuries and inflammations (Watsky et al., 1995) (Fig. 1, p. 14). The precorneal surface is covered with tear film and aqueous humor is on the other side. The corneal mean thickness in human and rabbit is 0.52 mm and 0.40 mm, respectively and it can be divided into five well-differentiated regions (Fig. 2).

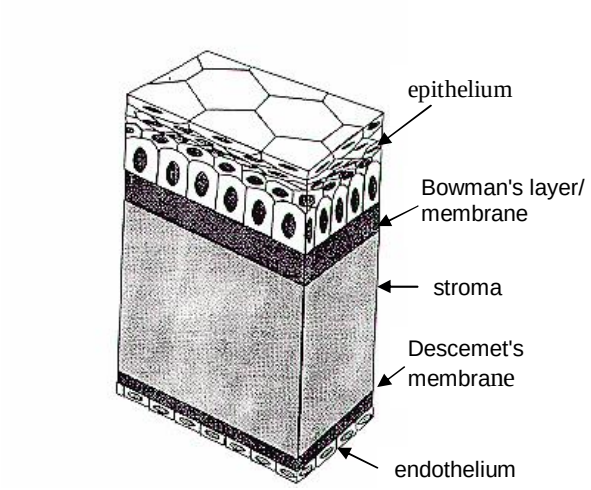

Figure 2. Illustration of the cornea (revised from Sasaki et al., 1999).

The corneal *epithelium* is typically five to seven cell layers thick, consisting of two layers of the flattened superficial cells, the multilayered polyglonal-shaped wing cells and one layer of columnar basal cells. The superficial cells are encircled by numerous tight junctional complexes and wing cells are attached to both superficial cells and to one another by desmosomes (Watsky et al., 1995; Sunkara and Kompella 2003). In addition both wing and basal cells have gap junctions between cells. The corneal epithelium lies on a thin layer of extracellular matrix (ECM) so called basement membrane which plays a crucial role in epithelial adhesion to the underlaying stroma. The epithelium is so called 'tight' epithelium with transepithelial electrical resistance (TER) of ~1000 $\Omega x cm^2$ (Rojanasakul et al., 1992) and the most apical cells alone

contribute to over half of the total resistance of the cornea (Klyce 1972). The mean thickness of the epithelium in humans is approximately 50 μm, and most of the apical layer contains microvilli (Reinstein et al., 1994; Watsky et al., 1995).

Acellular *Bowman's layer/membrane* is composed mainly of different collagen types (Nakayasu et al., 1986; Marshall et al., 1991). The layer does not regenerate and it is disorganised in the rabbit cornea. *Stroma* represents 90% of the thickness of the cornea, and it is composed mainly of hydrated type I collagen. Differentiated keratocytes are situated throughout the stroma and they can for example synthesize new collagen for tissue repair (Watsky et al., 1995). *Descemet's membrane* is the basal lamina of the endothelium (Johnson et al., 1982). The *corneal endothelium* is a single layer of unrenewable hexagonal cells in humans covering the posterior surface of the cornea and facing the anterior chamber (Sasaki et al., 1999).

2.2 Differentiation of corneal epithelium

2.2.1 Natural differentiation *in vivo*

The terminally differentiated, superficial cells of the corneal epithelium are continuously shed. It is estimated that the turnover time of the corneal epithelium is 7 days (Watsky et al., 1995). As cells are shed, basal cells, which are the only epithelial cells capable of mitosis, move upward from the basal layer, and differentiate into wing cells and finally into superficial cells. New basal cells originate from corneal stem cells, which exist in the limbus on the border of the cornea and sclera (Boulton and Albon 2004).

2.2.2 Induction of differentiation

Proper cell differentiation *in vitro* should lead to the expression of phenotypic properties characteristic of the functionally mature cells *in vivo* (Freshney 1987). As differentiation progresses, cell division is reduced and eventually lost whereas synthesis of the differentiated product increases. It is known that when cells are removed from their usual environment, they and their progeny generally remain true to their original instructions (Alberts et al., 1994). This phenomenon is particularly very strong in primary cell cultures that are obtained directly from tissues.

Differentiation process and stage of differentiation in cell culture can be achieved by establishing cultivation conditions in a way that make the induction and maintenance of differentiation of the cells possible. Overall, the best results from *in vitro* cell culture are reached using conditions that mimic *in vivo* conditions with respect to temperature, oxygen and CO_2 concentration, pH, osmolality and nutrition (Hornof et al., 2005). There are three main parameters especially governing the control of differentiation of corneal epithelial cells; soluble factors, use of permeable support systems and an air-liquid interface. Table 1 shows commonly used soluble inducers and permeable support factors in corneal epithelial cell proliferating and differentiation process.

In addition, most corneal epithelial cell media include serum that contains growth factors, carrier proteins, cell protective agents, cell attachment factors and nutrients (Cartwright and Shah 2002). Despite the growth factors in serum, some extra growth factors and nutrients are added to media to increase proliferation and differentiation of cells. On the other hand, high serum concentrations have been noticed to disturb cell proliferation and differentiation (Kruse and Tseng 1993). Some corneal cell lines are nowadays grown in commercially available serum-free medium (Gibson et al., 2003; Mohan et al., 2003a; Robertson et al., 2005).

2.2.3 Markers of differentiation

Corneal epithelial cell differentiation can be indicated by the expression of one or preferably more marker properties. Table 2 (p. 20) shows some usual differentiation markers. These markers are often determined after isolation of primary epithelial cells from intact cornea or after immortalization of primary cells for ensuring the cells exhibit corneal epithelial cells.

Tight junctions with its characteristic proteins, and desmosomes and microvilli formation are particular markers for final differentiation of corneal epithelium with flattened topical cells (Ward et al., 1997; Chang et al., 2000; Mohan et al., 2003a; Reichl et al., 2004). Measurements of TER and permeability support the morphology findings. Recently desquamation of stratified epithelia was also used as a marker of terminal differentiation of cornea (Robertson et al., 2005).

Table 1. Inducers and their function in corneal epithelial cell differentiation.

Inducers		**Action**
Soluble	EGF BPE	stimulates cell migration, proliferation, synthesis of basement membrane/extracellular matrix components, increases healing wounds [1-3]
	TGF-α	stimulates cell proliferation and differentiation in serum-free medium [4]
	Insulin	exerts metabolic and mitogenic effects in the ocular surface [5]
	Transferrin	the major iron transporter protein [6]
	Selenium	prevents oxidative DNA damage [7]
	Hydrocortisone	improves cloning efficiency [8]
	DMSO	stimulates differentiation [9]
	Cholera toxin	increases intracellular cyclic AMP [10]
	Ca^{2+}	proliferation, differentiation [11]
	Filters	allow cells to grow in a polarized state, cells can be fed to the basolateral and/or apical side
Permeable-support	Laminin collagens fibronectin	corneal basement membrane components, attachment and differentiation of cells [12]
	3T3 fibroblasts	cell interactions, regulatory functions and provoke differentiation, secrete stimulatory factors [13, 14]
	Amniotic membrane	includes e.g. laminin, collagen and fibronectin [15]
Air-liquid interface		promotes differentiation [16, 17]

[1] Schultz et al., 1992; [2] Bennett and Schultz 1993; [3] Gibson and Inatomi 1995; [4] Castro-Muñozledo et al., 1997; [5] Rocha et al., 2002; [6] Alberts et al., 1994; [7] Saito et al., 2003; [8] Fresney 1987; [9] Santos et al., 2003; [10] Spangler 1992; [11] Kruse and Tseng 1992; [12] Ohji et al., 1993 and 1994; [13] Sun and Green 1977; [14] Tseng and Kruse 1990; [15] Koizumi et al., 2000; [16] Minami et al., 1993; [17] Ban et al., 2003a

Table 2. Differentiation markers of corneal epithelium.

Marker		**Ref.**
Keratins	64-kDa (K3) 55-kDa (K12)	[1-8]
Morphology	cell layers, the shape of the cells, microvilli, tight junctions, desmosomes	
Tight junction proteins	claudins, occludin, ZO-1, ZO-2, ZO-3	[8-10]
Metabolic enzymes	e.g. ALDH, cytocrome P450	[1-3], [6]
Other	cytokines, growth factors, karyotypic analysis, basement membrane components, transporters, efflux proteins	[1], [3], [8], [11], [12], [13], [14]

[1] Kahn et al., 1993; [2] Araki-Sasaki et al., 1995; [3] Offord et al., 1999; [4] Chang et al., 2000; [5] Geerling et al., 2001; [6] Hernández-Quintero et al., 2002; [7] Mohan et al., 2003a; [8] Robertson et al., 2005; [9] Yi et al., 2000; [10] Ban et al., 2003b; [11] Okamoto et al., 1995; [12] Zieske et al., 1992; [13] Kawazu et al., 1999a and b, 2006 ; [14] Vellonen et al., 2006

2.3 Corneal cell models *in vitro*

Models of corneal epithelium are usually established by growing corneal epithelial cells on collagen/laminin/fibronectin coated cell culture filters. Models of entire cornea are constructed step by step on cell culture inserts by successive growth of corneal epithelial cells, stromal cells with collagen and endothelial cells (Hornof et al., 2005). Both reconstructions have been developed using both primary and immortalised cells from different species. These models can be used for toxicity testing, transcorneal permeation and metabolism studies. In addition, the organotypic cornea constructs might be useful study of the response of the cornea/corneal epithelium to surgery, wound healing and transplantation.

2.3.1 Primary cell cultures

Primary corneal epithelial cells are obtained directly from different species. These cells are fresh, but the condition of the cells and their behaviour in primary cell culture is affected by the choice of starting material (MacDonald 1994). Terminally differentiated epithelial cells grow poorly while corneal basal and limbal cells retain

proliferative capacity and undifferentiated features. However, primary cultures are not optimal for *in vitro* use owing to their senescence after several passages and their biological variability. Corneal epithelial cells from human (Ebato et al., 1987 and 1988; Ohji et al., 1993 and 1994; Pancholi et al., 1998; Bockman et al., 1998; Geerling et al., 2001) and rabbit (Jumblatt et al., 1983; Lass et al., 1989; Hernández-Quintero et al., 2002; Wallace et al., 2005; O'Brian et al., 2006) have been used in studies of cell attachments and basement membrane components, cellular uptake and toxicity tests as well as effects of growth factors in epithelial proliferation and differentiation processes. In addition the primary cells have been used in growing the epithelium on the cell culture filters for permeability experiments (Kawazu et al., 1998; Chang et al., 2000). The use of corneal limbal stem cells has mainly been focused on transplantation and corneal surface reconstruction studies (Germain et al., 2000; Boulton and Albon 2004).

2.3.2 Immortalised cell cultures

Primary cells can be transformed using some chemicals or viruses to establish continuous/immortalised cells. However, these cells may have altered growth characteristics, become tumorigenic and secrete abnormal levels of proteases and cell surface markers. Furthermore, expression of many differentiated or tissue-specific enzymes have been decreased and permanent cell lines are more likely to have chromosomal abnormalities (MacDonald 1994). On the other hand immortalised cells can be grown continuously and they survive well in liquid storage.

HCE-T (10.014) - Primary human corneal epithelial cells were infected with Adeno 12-SV40 hybrid virus or transfected with plasmid RSV-T (Kahn et al., 1993). In appropriate cell culture conditions these cells form a three-dimensional, tissue-like differentiated morphology with proper keratin expression. In addition, intercellular junctions and other ultrastructural features, TER properties and fluorescein permeation were determined in stratified cultures (Ward et al., 1997). Studies of stress protein gene expression, laminins and cell surface receptors have used the cells grown as monolayers (Braunstein et al., 1999; Kurpakus et al., 1999; Song et al., 2001; Lang et al., 2003). The stratified differentiated cells have only been used in toxicity testing.

The *HCE* (SV-40-immortalised) human corneal epithelial cell line exhibits a cobble-stone-like appearance, desmosome and microvilli formation similar to normal corneal epithelial cells and it expresses cornea-specific cytokeratin (Araki Sasaki et al., 1995). HCE-cells as monolayer are for instance used to study the cytotoxicity (Saarinen-Savolainen et al., 1998; Huhtala et al., 2002 and 2003). These cells were used in developing human corneal epithelial cell culture model (HCE-model) in the present study.

CEP1 or CEP1-17-CL4 are SV 40 T antigen retroviral vector immortalised human corneal cells, that show typical cobblestone morphology, and expresses cytokines, growth factors and metabolic enzymes that resemble original tissue (Offord et al.,1999). CEP1 cells have been used in developing and improving the sensitivity of alternative eye irritation tests (Debbasch et al., 2005). Thus far these cells have not grown on filters and so the formation the cell layers with desmosomes and tight junctions as well as permeability features are unknown.

HPV16-E6/E7 corneal cell line was developed by transfecting human primary corneal epithelial cells with tetracycline-responsive human papilloma virus (HPV)16-E6/E7 (Mohan et al., 2003a). The immortalised cells show typical corneal epithelial cell morphology, express tissue specific keratins, the cells form multilayered stratified cultures with surface microvilli and desmosome formation between cells. In addition, fluorescein permeation was determined. However, more specific profile of drug permeabilities and physical barriers are unknown.

Two different immortalised human cell lines from primary cultures of human corneal epithelial cells infected with a retroviral vector encoding human telomerase reverse transcriptase (*hTERT*) have been developed (Gipson et al., 2003; Robertson et al., 2005). These cell lines exhibit well-stratified cell layers with differentiation keratin markers. Permeability features of these cells have not been evaluated.

SIRC-cells (Statens Seruminstitut rabbit corneal cells) are continuously grown cells, which have been widely used during the last three decades in dozens of studies of corneal transport and permeability (Tak et al., 2001; Dey et al., 2003; Talluri et al., 2006) and toxicology (North-Root et al., 1982; Scuderi et al., 2003), although it has been shown that SIRC-cells are fibroblastic cells (keratocytes) and not corneal epithelial

cells (Niederkorn et al., 1990). These cells grow as monolayers, and form a tight barrier (Goskonda et al., 1999). The model has been found to predict the permeability of ophthalmic drugs across corneal membranes (George et al., 2000a).

IHCEC immortalised corneal epithelial cells are used in commercially available human corneal epithelial model for *in vitro* toxicology testing (SkinEthic Laboratories, Nice, France). IHCEC-cells are cultivated in chemically defined medium on permeable polycarbonate inserts at air-liquid interface (Nguyen et al., 2003). Histologically, cultures appeared as a multilayered, stratified epithelium resembling human corneal epithelium while desmosomes, hemidesmosomes, laminin and keratin expression was also identified. The use of this model has focused on toxicity and eye irritation studies (Doucet et al., 2006; Van Goethem et al., 2006). Permeability features have not been studied.

Animal corneal epithelial cells- Immortalised rabbit corneal epithelial cell lines by Araki et al. (1993) and Okamoto et al. (1995) express cornea specific keratin, microvilli and intercellular desmosomes. Immortalised rabbit corneal cells have been used in developing stratified epithelium *in vitro* (Yang et al., 2000; Burgalassi et al., 2004).

The RCE1-cell line is a rabbit corneal epithelial cell line that was developed by maximizing the number of passages of primary rabbit corneal epithelial cells in the presence of additives that are stimulators of epithelial growth (Castro-Muñozledo 1994). The culture stratified and expressed specific keratin pairs. Immortalised cell lines have also been established from rat (Araki et al., 1994) and hamster (Halenda et al., 1998). None of these cell lines have been used in studies of epithelial barrier features.

2.3.3 Whole cornea models

The first human corneal equivalents comprising epithelium, stroma and endothelium were constructed using immortalised human corneal cells (Griffith et al., 1999). Engineered corneas mimicked human corneas in morphology, biochemical marker expression, transparency, ion and fluid transport, and gene expression. Another three-dimensional corneal equivalent was constructed recently using SV40-immortalised human corneal epithelial cells (HCE), human corneal keratocytes (HCK) and human

corneal endothelial cells (HCEC) (Zorn-Kruppa et al., 2005). This model was developed as a replacement for eye irritation tests (Engelke et al., 2004).

Human corneal construct (HCC) includes monolayer of immortalised endothelial cells (HENC), native keratocytes mixed with type I rat-tail collagen in the middle and on the top immortalised epithelial cells (CEPI-17-CL-4) (Reichl et al., 2004 and 2005; Meyer et al. 2005). This whole cornea model was used in permeability studies to determine the transcorneal drug transport of different nanosuspensions (Friedrich et al., 2005).

The primary corneal cells from bovine (Minami et al., 1993; Tegtmeyer et al., 2001 and 2004; Reichl et al., 2003), rabbit (Zieske et al., 1994) and fetal pig (Schneider et al., 1997 and 1999) have also been used in developing corneal cell models.

2.4 Ocular drug absorption

2.4.1 Corneal route

Drug absorption from the surface of the eye can be either corneal or noncorneal (Fig. 1, p. 14). For most ocular drugs, passive diffusion is the main transport process across the cornea. Passive diffusion is influenced by molecular weight, partition coefficient, and degree of ionization of the drug. The corneal epithelium is the main limiting barrier for hydrophilic drugs that penetrate through the paracellular pathway. The corneal surface epithelial intercellular pore size has been estimated to range between 20 Å (Hämäläinen et al., 1997b) and 30 Å (Tonjum 1974; Lee et al., 1986) and only very small ionic and hydrophilic molecules penetrate corneal epithelium paracellularly. The corneal epithelium is negatively charged and because the isoelectric point is 3.2, the paracellular space is more permeable to cations than to anions at physiological pH (Rojanasakul and Robinson 1989; Liaw et al., 1992).

Most drugs that are used clinically are sufficiently lipophilic to permeate across the cornea via transcellular route (Sasaki et al., 1999). Drug lipophilicity is one of the most important factors and it has been reported that the log (partition coefficient) of 2-3 is optimal for corneal penetration (Schoenwald and Ward 1978; Huang et al., 1983; Schoenwald and Huang 1983). In general, adjusting pH so that a drug is mostly in the unionized form increases its lipophilicity and thus, its transcellular permeability and

ocular absorption (Burstein and Anderson 1985; Mitra and Mikkelson 1988). The hydrophilic stroma represents a limiting barrier for penetration by highly lipophilic compounds. The corneal endothelium is lipophilic in nature (Huang et al., 1983). It is a 'leaky' barrier, which allows both paracellular and transcellular permeability of many drugs (Prausnitz and Noonan 1998).

Many ocular drugs interact with transporters, but not much is known about the expression and function of transporters in the corneal epithelium (Mannermaa et al., 2006). Functional P-glycoprotein (P-gp) has been identified in cornea and corneal cell lines (Kawazu et al., 1999b and 2006; Dey et al. 2003) and recently P-gp has also been found to be active *in vivo* by restricting topical erythromycin absorption across the cornea (Dey et al., 2004). Accordingly, recent preliminary studies showed multidrug resistance-associated protein (MRP) expression at RNA level in the cornea epithelium (Vellonen et al., 2006).

Drug metabolising enzymes in ocular tissues constitute a metabolic barrier that limits the ocular entry of xenobiotics. Various enzyme classes have also been found in corneal tissues (Duvvuri et al., 2004). The corneal epithelium has been shown to be metabolically most active for esterases (Lee et al., 1982; Lee 1983), aminopeptidases (Stratford and Lee 1985), ketone reductase (Lee et al., 1988) and N-acetyltransferase (Campbell et al., 1991). Esterases and ketone reductase are perhaps the most important ocular drug-metabolising enzymes due to their role in the activation of prodrugs or soft drugs.

2.4.2 Noncorneal route

The noncorneal route involves penetration across the conjunctiva and sclera into the intraocular tissues. The conjunctiva is a relative leaky membrane with rich blood flow and a large surface area (Watsky et al., 1988). Despite these properties this pathway does not appear to be important in drug absorption for most ocular drugs, but the route has been shown to be particularly important for hydrophilic compounds with large molecular weights (Ahmed and Patton 1985 and 1987; Chien et al., 1990; Hämäläinen et al., 1997a and b). Drug absorption through conjunctiva is influenced less by the

molecular size and lipophilicity than in the cornea. In general, sclera shows higher permeability than the cornea and conjunctiva.

Conjunctival uptake via its blood vessels and solution drainage via the nasolacrimal duct may lead to systemic absorption. In addition, drainage of tears and instilled solutions away from the front of the eye increase the precorneal loss of the drug (Patton and Robinson 1976).

2.5 Transfection of the cornea

The cornea is an attractive target for gene therapy owing to its simple histological structure, an immuno-priviledged nature, and easy accessibility (Jun and Larkin 2003). Furthermore, the cornea allows local application of therapeutic agents with reduced risk of systemic effects and many animal models for human ocular diseases have been developed. Accordingly, the function and health of the eye can be evaluated non-invasively and in quantitative fashion, and the eye can be directly observed and followed for signs of disease and inflammation (Bennett and Maguire 2000; Borrás 2003; Jun and Larkin 2003). Corneal gene therapy studies are mainly focused on endothelium to prevent allograft rejection after corneal transplantation and different injections into the eye are the most common administration methods used *in vivo*. Gene therapy of epithelial layer has attracted less attention. Because it is practically impossible to transfer genes systemically to the eye, the delivery has been achieved mainly through injections using viral-based vectors (Table 3, p. 28). However, topical delivery is another useful but less investigated delivery method for transgenes.

Transgene expression is usually monitored using reporter molecules such as chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), luciferase (luc) and green fluorescent protein (GFP) (Hiramatsu et al., 2005). β-galactosidase has mainly been used in demonstrating the localization of transgene expression in cells and tissues, whereas other reporter genes express time dependent quantitative estimation of reporter gene expression. However, previously mentioned assay methods of the secreted proteins require tissue sampling and protein extraction, which is inconvenient for pharmacokinetic determinations in *in vivo* experiments. Furthermore, for the analysis of protein expression in tear fluid and aqueous humor, the aforementioned reporter

molecules are not practical or they are impossible to use. Moreover, it is known that gene transfer vectors that function well *in vitro* may not function *in vivo*; protein expression substantially decreases or the expression is not seen at all. However, in screening different transfection vectors differentiated cell cultures *in vitro* have been in minor use as a linkage between dividing cells *in vitro* and differentiated cells *in vivo*.

Transfection of therapeutic genes has also increased in recent years. In these cases, the success of the transfection is seen by the improved condition of the eyes.

2.5.1 Viral vectors

Viruses have developed efficient strategies to penetrate into host cells, transport their genetic information into the nucleus either to become part of the host's genome or to constitute an autonomous genetic unit (Pfeifer and Verma 2001). Virus vectors are designed by identifying the viral sequences that are required in assembling of viral particles, packaging of the viral genome into particles and delivering the foreign gene to the target cells via cell surface receptors (Kao 2002). Dispensable genes are deleted from the viral genome, and the residual viral genome and transgene are integrated into the vector construct. Viral vectors are divided into integrating and nonintegrating vectors, which are capable of permanent and temporary expression of the transgene, respectively.

Successful virus mediated corneal transductions have been performed *in vivo* (Table 3, p. 28), *ex vivo* with isolated corneas and *in vitro* with different corneal cells from different species (Jun and Larkin 2003; Rosenblatt and Azar 2004; Mohan et al., 2005).

Adenovirus and adeno-associated virus (AAV) are DNA viruses, which are capable of infecting both dividing and non-dividing cells (Mohan et al., 2005). AAV is a non-pathogenic virus, which integrates into host cell genome and thus, is capable of producing long-term gene expression genome. Other DNA-viruses, such as herpes simplex viruses (HSV) and baculovirus can infect non-dividing cells (Das and Miller 2003).

Table 3. Examples of transductions into cornea *in vivo*.

Vector	Transgene	Administration / animal	Protein or mRNA expression / response
AAV [1-4]	VEGFr, GFP, ß-gal	anterior chamber injections / rats and rabbits	endothelium, reduced neovascularisation
	EGFP	topical application after scraping off superficial epithelial cells / rats	epithelium
	CAT, ß-gal	lamellar flap-technique / rabbits	keratocytes
Adenovirus [3, 5-9]	HO-1	anterior chamber injections / rabbits	epithelium, endothelium
	ß-gal, GFP	anterior chamber injections / rabbits and monkeys	endothelium
	ß-gluc, ß-gal	anterior chamber injections, inside stroma with lameller keratotomy / mice	endothelium, keratocytes, reduced corneal clouding
	EGFP	topical application after scraping off superficial epithelial cells, intrastromal injections / rats	epithelium, stroma
Lentivirus [3, 10-12]	GFP	anterior chamber injections / mice	endothelium
	EGFP	topical application after scraping off superficial epithelial cells / rats	epithelium
	EGFP	intravitreal injections / mice	endothelium
Retrovirus [13,14]	ß-gal HStk dnG1	topical application after a superficial keratectomy / rabbits	keratocytes, development of corneal haze was inhibited
HSV [15]	ß-gal	intracameral and intravitreal injections, topical application after corneal scarification / rats and mice	entire cornea
Baculovirus [16]	GFP	intravitreal injections / mice	corneal endothelium

[1] Lai et al., 2002; [2] Tsai et al., 2002; [3] Igarashi et al., 2002a and b; [4] Mohan et al., 2003b, [5] Abraham et al., 1995; [6] Borrás et al., 1996; [7] Borrás et al., 2001; [8] Kamata et al., 2001; [9] Carlson et al., 2004; [10] Bainbridge et al., 2001; [11] Challa et al., 2005; [12] Takahashi et al., 2002; [13] Seitz et al., 1998; [14] Behrens et al., 2002; [15] Spencer et al., 2000; [16] Haeseleer et al., 2001

Retrovirus and lentiviruses are RNA viruses, which cause long-term expression due to chromosomal integration (Das and Miller 2003; Rosenblatt and Azar 2004; Mohan et al., 2005). Retrovirus provides gene expression for the lifetime of the cell, but infect only dividing cells whereas lentiviruses are capable of transfecting non-dividing cells.

Disadvantages of the use of viral vectors include limitations on the size of therapeutic genes, random integration in the human genome, insertional mutagenesis, immunogenicity against virus as well as infections and inflammations in the eye.

2.5.2 Non-viral methods

Non-viral gene delivery systems consist of a therapeutic gene and a synthetic gene delivery system. Non-viral vectors are also called plasmid-based gene expression systems, because a transfected therapeutic/marker gene and other DNA sequences to control the production of the resultant protein, are inserted into a plasmid-DNA vector. Plasmids are large, hydrophilic macromolecules with a net negative surface charge, which prevent plasmids to cross biological membranes efficiently (Mahato et al., 1997). Thus a carrier system is needed to transfer plasmid DNA (pDNA) across the cell membranes into cells. Depending on the carrier method, non-viral methods can be classified in physical and chemical methods.

Non-viral vectors are relatively safe, capable of the transfer of large genes, non-inflammatory, non-toxic and non-infectious (Mohan et al., 2003b). In addition, they can be designed based on characterized agents, they are not limited by the size of the DNA, their production is inexpensive, and they can be produced in large quantities (Das and Miller 2003). Furthermore both dividing and non-dividing cells can be tranfected using non-viral methods. On the other hand, non-viral vectors have low transfection efficiency, relatively poor transgene expression and they are capable only in transient transfection.

Physical methods- In the *gene gun method* gold microparticles are coated with naked pDNA and the DNA is delivered into the target cells/organ using explosive or gas-driven ballistic devices. This method allows direct penetration through the cell membrane into the cytoplasm and even the nucleus (Niidome and Huang 2002). *Electroporation* uses electric field pulses, which cause transient and reversible pores in

the plasma membrane of cells, and drive the negatively charged DNA into the cytoplasm (Blair-Parks et al., 2002; Trezise et al., 2003). Before electric field pulses pDNA construct has to be injected into target cells/tissue. However, the disadvantages of these methods are a low transfection efficiency and possible tissue damage and cell death (Kao 2002). Furthermore, injection of *naked pDNA* and the use of *ultrasound* as a transgene delivery method have been studied in transfection of cornea (Angella et al., 2000; Stechschulte et al., 2001; Sonoda et al., 2006). Some corneal transfections *in vivo* using physical methods are illustrated in Table 4 (p. 32).

Chemical methods consist of an expression cassette, inserted into a plasmid and complexed with positively charged cationic lipid, cationic polymer, or a mixture of these (Lechardeur et al., 2005). In addition, various forms of receptor-mediated gene transfer are used (Varga et al., 2000). The functions of the various types of synthetic gene carriers are to condense and protect pDNA from premature degradation during storage and transportation and to augment DNA delivery into the cell nuclei (Mahato 2005). Efficiency and safety of the transfection reagents are strongly dependent on the lipid:DNA ratios and concentrations; decreased lipid concentrations reduce toxicity and efficiency (Dannowski et al., 2005).

The delivery of pDNA into the cell includes cellular binding and uptake, endosomal escape and nuclear delivery. DNA release from the complex begins by binding the positively charged DNA/carrier complex to, for example negatively charged glycosaminoglycans (GAGs) on the target cell surface membrane (Ruponen et al., 2004). After that the complex is endocytosed into endocytic vesicles (endosomes) of the cell (Clark and Hersh 1999; Lechardeur et al., 2005). The size and the composition of the complex, as well as cell surface properties and endocytic activity of the specific cell type influence the internalization pathways (Khalil et al., 2006). According to present knowledge DNA has to release from the complex before transcription in nucleus. However, it is not known if DNA release from the complex takes place in the endosomes, cytoplasm and/or nucleus.

Cationic lipids, such as DOTAP are amphiphilic molecules that interact with the negatively charged phosphate backbone of DNA, neutralizing the charge and promoting the condensation of DNA into more compact structure (Mahato et al., 1997). The

cationic lipids have one or more hydrophobic acyl chains, possible linker group, and a positively charged headgroup, which interacts with plasmid. The addition of a lipid-like compound or neutral lipid, like DOPE, is typically used as co-lipid to facilitate the release of plasmid DNA from endosomes after endocytic uptake of the pDNA/liposome complexes (Farhood et al., 1995). Furthermore, the ratio of DNA to lipid influences the transfection efficiency; charge ratios (+/-) higher than one are preferred (Tseng et al., 1997). Liposomes have been successfully used in delivering genes into immortalised and primary corneal cells of different species *in vitro* (Pleyer et al., 2001; Nguyen et al., 2002; Bertelmann et al., 2003; Dannowski et al., 2005), organ-cultured cornea *in vitro* (Klebe et al., 2001) and endothelium *ex vivo* (Arancibia-Cárcamo 1998; Nguyen et al., 2002). Examples of the transfections *in vivo* are illustrated in Table 4 (p. 32).

Cationic polymers, such as PEI and dendrimers, with a strong positive surface charge, make them suitable to bind and package large negatively charged pDNA. PEI and dendrimers have been shown to mediate transfection in various cell lines *in vitro* (Haensler and Szoka 1993; Boussif et al., 1996), whereas the transfection efficiency *in vivo* is much less. Overall, PEI (or any cationic polymer) has been only once used *in vivo* for the corneal transfections (Kuo et al., 2005). In addition, human corneal endothelium expressed the transgene after transfection with polyamidoamine dendrimers *ex vivo* (Hudde et al., 1999).

In receptor mediated gene delivery pDNA-vector complex is targeted to a particular target molecule on the cell surface. This has the potential for specific delivery to particular cells, and also delivery to molecules that are optimal for gene delivery (George et al., 2000b). Transferrin-PEI conjugate (Tf-PEI) system (Nguyen et al., 2002), the similar transferrin-mediated lipofection method (Tan et al., 2001) and integrin-targeted peptide/pDNA complexes (Shewring et al., 1997) have been tested to deliver the transgene into rabbit, human and pig endothelial cells *in vitro*. Coupling antibodies to lipid-DNA complexes leads to the production of *immunoliposomes*, and this antibody targeted gene transfer method was used to transfer genes into primary human corneal endothelial cells *in vitro* and *ex vivo* (Tan et al., 2003). Polyethylene glycols (PEGs) stabilize the liposomes and PEGs were used in conjugating the surface of liposomes in immunoliposomes in intravenous gene transfer (Zhu et al., 2002; Zhang

et al., 2003). In these studies transgene expression was seen even in the epithelium of cornea in mice and rhesus monkey.

Table 4. Examples of non-viral transfections into cornea *in vivo*.

Vector	**Transgene**	**Administration / animal**	**Protein expression/ response**
Electro-poration [1-4]	ß-gal tPA	anterior chamber injections / rats	endothelium, fibrin formation decreased
	GFP, luc	injections into stroma, intracorneal and subconjunctival injections / mice and rats	epithelium stroma
Gene gun [5-8]	EGFP, luc, IL-4, IL-10, CTLA4	cornea / mice	epithelium prolonged corneal graft survival
	GFP, ß-gal, HA, OGFr,	cornea / rabbits and rats	epithelium
Liposomes [9-13]	ß-gal	topical application, injections into anterior chamber, topical application / rats	epithelium
	CAT, ß-gal	lamellar flap-technique / rabbits	stroma
	luc	intravitreal injections / rabbits	cornea, aqueous humor
	EGFP, BAI1-ECR	subconjunctival injections / rabbits	stroma, reduced neovascularization
PEI [14]	GFP, b-FGF	injections into stromal pocket / rats	epithelium keratocytes induced angiogenesis
Immuno-liposomes [15,16]	ß-gal	injections intravenously / mice and rhesus monkeys	epithelium

[1] Oshima et al., 1998; [2] Sakamoto et al., 1999; [3] Blair-Parks et al., 2002; [4] Oshima et al., 2002; [5] Tanelian et al., 1997; [6] Shiraishi et al., 1998; [7] König et al., 2000; [8] Zagon et al., 2005; [9] Masuda et al., 1996; [10] Matsuo et al., 1996; [11] Mohan et al., 2003b, [12] Kawakami et al., 2004; [13] Yoon et al., 2005; [14] Kuo et al., 2005; [15] Zhu et al., 2002; [16] Zhang et al., 2003

3 AIMS OF THE STUDY

The overall aim of the present study was to develop a cell culture model of human corneal epithelium for drug permeability studies. The specific aims of the study were:

1. To develop appropriate cultivating conditions for SV40 immortalised human corneal epithelial cell line to generate differentiated corneal epithelium for drug permeation studies.

2. To characterise the morphology, transepithelial electrical resistance, and permeability of HCE-model using model compounds with different lipophilicity, molecular size and charge.

3. To evaluate the suitability of HCE-model for transfection studies.

4. To study the transfection of the corneal epithelium and its use as a platform for transgene product secretion into the lacrimal fluid and anterior chamber.

4 MATERIALS AND METHODS

4.1. Cell culture (I)

Immortalised human corneal epithelial cells (HCE SV-40-immortalised; Araki-Sasaki et al., 1995) were seeded on polyester and polycarbonate cell culture permeable membrane filters, which were coated with corneal basement membrane components; rat tail collagen type I or mouse laminin. Some filters were coated with collagen mixed with mouse embryonic fibroblasts to mimic corneal stroma. Filters without any coating were also used. The cells were grown using standard culture medium both in the apical and the basolateral chambers until the cells were confluent. Then, the cells were exposed to an air-liquid interface for 2-3 weeks. The culture medium was replaced every other day.

4.2 Transepithelial electrical resistance - TER (I)

TER was measured by Endohm^{TM} at different phases of cell growth. Measurement was based on the voltage difference while the current is passed through cell layers.

4.3 Histology (I)

Morphology such as number of cell layers and the shape of cells were analysed using light microscopy. Different cell organelles and junctions between cells were visualised with transmission electron microscopy (TEM), which is based on electrons which pass through specimen and are scattered by structures stained with the electron-dense material.

4.4 Permeation studies (I and II)

HCE-model- The transport of model probes across cell culture were determined using ^{3}H-mannitol and 6-carboxyfluorescein (6-CF) as hydrophilic markers for characterising the paracellular permeation between the epithelial cells. Rhodamine B was used as lipophilic marker for establishing transcellular permeability of HCE-culture.

Eight β-blockers (atenolol, sotalol, nadolol, pindolol, timolol, metoprolol, propranolol and alprenolol) with logP values ranging between -0.62 and 3.44 were used to study the influence of lipophilicity on drug permeation across the HCE-cell layer. Permeation

studies with polyethyleneglycols (PEGs; mean molecular weights of 200, 400, 600, and 1000) were carried out to characterise the effects of molecular size on permeability and to determine the paracellular pore size and porosity of the differentiated HCE-cells. Positively charged amino-polyethylene glycols (amino-PEGs) with mean MW of 350-750 (a gift from Dr. Etienne Schacht, University of Ghent) were used to study effects of size and charge on the passive paracellular permeation across differentiated HCE-cells.

Esterase activity of the differentiated HCE-cells was examined with the permeation of fluorescein and fluorescein diacetate across cells. This method is based on the esterases of cells which are able to hydrolyse fluorescein diacetate to fluorescein.

Excised rabbit corneas were used to compare the drug permeabilities and esterase activity of the intact tissue with the HCE-model. All animal experiments conformed to the ARVO Resolution on the Use of Animals in Research. The cornea of rabbit was dissected with a scleral ring and the permeation studies were performed using Snapwell side-by-side diffusion chambers.

4.5 Gene transfer into corneal cells (III)

In vitro transfection- Complexes were performed with pCMV-SEAP2 and DOTAP/DOPE with or without PS at charge ratios +/-2 and +/-4, and PEI at charge ratio +/-8. The cells were transfected at three stages of differentiation; the next day after seeding of the cells onto filters (dividing cells), one week after seeding the cells were exposed to air-liquid interface (dividing/differentiating cells), and after 4-5 weeks at the stage of differentiation (differentiated cells). After transfection samples were withdrawn daily for one week. The condition of the differentiated cells was examined daily by TER measurement.

SEAP permeation across HCE-layer was followed to identify the degree of permeation of the large protein molecule across differentiated HCE-cells.

In vivo transfection- Complexes of DOTAP/DOPE/pCMV-SEAP2 (+/- 2), naked pCMV-SEAP2, DOTAP/DOPE/pSEAP2-Basic (+/- 2) and DOTAP/DOPE/ pCMV-Luc4 (+/- 2) were applied topically to the male albino New Zealand rabbits. The amount of pDNA delivered was 24 μg per eye and the treatment was repeated twice. Precorneal tear fluid was withdrawn daily from each eye for the first four days and on the seventh

day after transfection. Aqueous humor samples were taken from the anterior chamber with needle under microscope from anaesthesised rabbits after 1, 2 and 3 days following transfection.

4.6 Analysis of model compounds

The radioactivity and fluorescence were measured using a liquid scintillation counter and fluorescence plate reader, respectively (**I** and **II**).

PEGs and amino-PEGs were quantified using the combination of reversed-phase high performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS). The HPLC-ESI-MS method is described by Palmgrén et al., (2002). Shortly, the samples of PEGs and/or charged PEGs were driven into lipophilic HPLC column. The retention times in the column were dependent on the oligomer molecular weight as they are eluted at different concentrations of acetonitrile in the gradient mobile phase. Liquid eluent with separated PEGs and aminoPEGs was sprayed through the electrospray needle and as a result every charged drop contains only one PEG oligomer. Solvent evaporated from drops and PEG molecules were transmitted into MS-detector for quantification.

β-blockers were analysed simultaneously by gradient HPLC with combined UV and FL detection as described earlier (Ranta et al., 2002). A reversed phase column was also used in this method. Ultraviolet (205 nm) and fluorescence detection with 230 nm excitation and 302 nm emission filters were used.

The fluorescence-based real-time reverse transcription PCR (real-time RT-PCR) was used for the quantification levels of steady-state luciferace mRNA produced in corneal epithelial and conjunctival cells (**III**).

SEAP-assay- Great EscAPE SEAP Chemiluminescence Detection kit was used to determine SEAP from the cell culture medium, tear fluid and aqueous humor samples following manufacturer introductions. The detection of SEAP was performed with luminometer (**III**).

4.7 Data and statistical analysis

Apparent permeability coefficients (P_{app}) in cm/s of the cultured HCE-cells and filter together or excised cornea were calculated using the equations illustrated in article **I**. The effects of filter and extracellular matrix on drug permeation (P_{filter}) were taken into account in determining the permeability of the cultured corneal epithelium (P_{cell}) without support.

*Paracellular space dimensions **(II)*** - Paracellular permeability data (P_{cell}–values of PEGs) and effusion-like analysis allow estimation of the size and number of the paracellular pores in the biomembranes. An effusion-based theory assumes that the low probability of finding the pore, rather than diffusion, determines the paracellular permeation. Increasing molecular size of the permeant decreases the rate of paracellular penetration. Paracellular pore size and porosity in HCE-model was obtained from effusion based equations from Hämäläinen et al. (1997a and 1997b).

Mann-Whitney's U-test and paired two-tailed t-test were used to test for statistical significances. $P < 0.05$ was taken to represent statistical significance in both analyses (**I** and **III**).

Pharmacokinetic parameters of SEAP expression after transfection *in vitro* and *in vivo* were obtained using equations illustrated in the article **III**.

5 RESULTS

5.1 Differentiation

Based on light microscopy and TEM, the HCE-cells consisted of 5-8 cell layers when they were grown on collagen, collagen/laminin or collagen/fibroblasts coated membrane filters (**I**; Fig. 3 B). The most apical cells were flat with tight junctions, microvilli and desmosomes (TEM) (**I**; Fig. 4). The thickness of the cultured corneal epithelium was found to be 70 µm which is close to the thickness of the human corneal epithelium (50-70 µm) (Watsky et al., 1995). When the cells were grown on polycarbonate filters, 3-10 cell layers were formed without flattening of the apical cell layers (**I**; Fig. 5). However, there were some tight junctions, microvilli and desmosomes. If the cells were cultivated without the air-liquid interface, only 2-3 cell layers without flattened cells and tight junctions were formed (**I**; Fig. 6).

The TER-values of HCE-cells without the air–liquid interface were ~ 100 - 200 Ωxcm^2 and the P_{cell} of ^{3}H-mannitol was (10 – 20) x 10^{-6} cm/s, regardless of the filter or coating material used. TER-values remained at the same level even after the cells reached confluence in one week after seeding them onto the filters. Under the air-liquid interface the TER increased for 2-3 weeks to ~200 – 800 Ωxcm^2 depending on the culture conditions (**I**; Fig. 1). With increasing TER the permeability coefficient decreased. At TER values of 400-800 Ωxcm^2 the P_{cell} values were (1 – 2) x 10^{-6} cm/sec (**I**; Fig. 2). TER values were similar before and after the permeability experiment.

The cells migrated through the filter with 3 µm pore size. In each coating system the polyester filters were better than polycarbonate filters for culturing HCE barrier. Polyester filters (0.4 µm) coated with collagen or laminin alone, or mixture of collagen/fibroblasts or collagen/laminin were the best methods for culturing HCE cell layers.

5.2 Paracellular and transcellular permeability

Rhodamine B penetrated across the cell layers 21 and 11 times faster than hydrophilic 6-carboxyfluorescein or ^{3}H-mannitol, respectively (**I**; Table 1). In excised rabbit cornea the differences for the same comparisons were 39- and 48-fold, respectively. HCE cell

culture model was more permeable to ^{3}H-mannitol than isolated rabbit cornea ($P < 0.05$), while in the case of rhodamine B and 6-carboxyfluorescein no significant differences were seen.

Permeability of PEGs in the HCE-model decreased with increasing molecular weight from 1.20 x 10^{-6} cm/s of PEG_{282} to 0.50 x 10^{-6} cm/s of PEG_{942} (**II**; Fig. 1). Permeabilities of PEGs in the HCE-model are 1.6 to 2.3 times greater than in isolated rabbit cornea (data of Hämäläinen et al., 1997a). The relative difference between the isolated cornea and culture model increases with increasing molecular weight. For example the ratio of the permeabilities (HCE-model/cornea) at MW ~ 300 and 800-900 were about 1.5 and 3.0, respectively.

The relationship between the permeability (P_{cell}) and the radius of PEG oligomers in the HCE-cell model and excised cornea shows a linear relationship and intercepts on the x-axis at positive values (**II**; Fig. 2). Porosity, pore size and the number of pores/cm^2 in the differentiated HCE-model and excised rabbit cornea were similar, but not identical. In both cases, the pore radius ranged between 0.7 and 1.6 nm and the porosity in terms of paracellular pores was in the range of (1 – 3) x 10^{-7} (**II**; Table1).

P_{app} of amino-PEGs are illustrated in Fig. 3 (unpublished data). Permeability of amino-PEGs across the HCE-model also decreased with increasing molecular weight from 0.7 x 10^{-6} cm/s of amino-PEG_{251} to 0.23 x 10^{-6} cm/s of amino-PEG_{955}. The P_{app} values of amino-PEGs are at lower level than the values of uncharged PEGs.

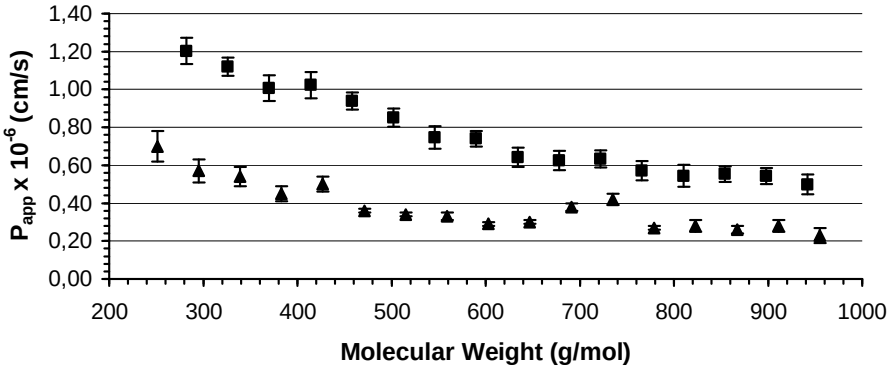

Figure 3. The permeability (P_{app}) of PEGs (squares) (**II**; Fig. 1) and amino-PEGs (triangles) in the HCE-model (n = 6-9; mean ± S.E.M.).

Permeability of β-blockers in the HCE-model increased with increasing lipophilicity according to a sigmoidal relationship (**II**; Fig. 3). In the HCE-model permeability coefficient of the most hydrophilic drug, sotalol (P_{app} 1.21 x 10^{-6} cm/s) was 13.5 times smaller than the permeability of the most lipophilic drug, betaxolol (P_{app} 15.01 x 10^{-6} cm/s) (**II**; Table 2). The difference was 38.6-fold in the isolated rabbit cornea (Wang et al., 1991; Prausnitz and Noonan, 1998). Experiments did not show any preferable directionality suggesting lack of active transport of the permeants in the differentiated HCE-cells.

Fluorescein diacetate showed improved permeability in the HCE-model (49-fold) and in the rabbit cornea (31-fold) compared to the permeability of fluorescein. These data show that the esterases cleave acetates from fluorescein diacetate efficiently in the HCE-model and rabbit cornea.

5.3 Transfections

In vitro transfections- Overall the highest rate of SEAP secretion was during the second and the third day after transfection and levels of SEAP secretion decreased clearly after days 2 and 3 in all gene transfer experiments.

PEI +/- 8, DOTAP/DOPE +/- 2, DOTAP/DOPE/PS at charge ratio +/- 2 and +/- 4 are among the most effective carriers in the dividing cells (**III**; Fig. 3A and B). SEAP secretion was 1.5-2.1 times higher to the apical than to the basolateral side.

PEI is the most effective carrier in transfections of the dividing/differentiating HCE-cells (**III**; Fig. 4A). The secreted amount of SEAP is at least 1.7-fold greater than with the other carriers. Peak concentration of SEAP after PEI mediated transfection is 14 times lower in the dividing/differentiating than in the dividing cells.

DNA complexes of the DOTAP/DOPE +/-2, DOTAP/DOPE/PS +/-2 and DOTAP/DOPE +/-4 have similar transfection efficacy in differentiated HCE-cells (**III**; Fig. 4B). Peak concentration of SEAP after DOTAP/DOPE +/-2 transfection with differentiated cells is 196 and 8.2 times lower than in the dividing and dividing/differentiating cells, respectively. PEI +/-8 did not mediate any detectable SEAP transfection in the differentiated cells.

During 5 hours of permeation experimentation approximately 14.1% of SEAP was transported across the filter alone, and less than 0.01% could permeate across the cultured differentiated HCE.

In vivo transfections- In tear fluid, peak SEAP concentrations were reached 1-2 days after topical instillation of DOTAP/DOPE/pCMV-SEAP2 ($n = 6$; $p < 0.05$) and the concentration of SEAP remained statistically significant during the third and fourth days (**III**; Fig. 5). Transfection with naked-DNA did not cause statistically significant expression of SEAP in the tear fluid of rabbits.

SEAP concentration in aqueous humor varied from (1.4 ± 0.4) ng/ml to (4.7 ± 1.4) ng/ml ($n = 14$-20) within three days after transfection and was significantly higher compared to situation before transfection ($p < 0.05$). The average steady-state (C_{ss}) concentration of SEAP protein in aqueous humor within three days after transfection was 3.05 ng/ml. No SEAP was detected in aqueous humor after transfection with naked pDNA.

Secretion rates- Secretion rate of SEAP to the basolateral side of differentiated HCE-cells was (0.51 ± 0.09) - (1.63 ± 0.18) ng/h/cm^2. *In vivo* the mean rate of SEAP secretion to aqueous humor was (0.20 ± 0.06) - (0.68 ± 0.20) ng/h/cm^2 during the three days after transfection. Secretion rates of SEAP into the tear fluid and aqueous humor were (0.12 ± 0.04) - (0.30 ± 0.07) ng/h and (0.31 ± 0.09) - (1.08 ± 0.32) ng/h, respectively.

Real-time RT-PCR study shows that relative expressions of luc-mRNA from rabbit corneal epithelium and conjunctiva are about 10- and 20-fold higher than in the control tissues, respectively (**III**; Fig. 6). No luciferase expression at mRNA level was detected in these tissues after transfection with naked pDNA.

6 DISCUSSION

One of the main problems in development of new ophthalmic drugs is their poor absorption into the eye after topical application. The cornea is the main route of drug absorption from tear fluid to the inner eye for clinically used ophthalmic drugs. In order to reduce the number of animal experiments, new research methods for drug delivery studies are needed.

In the present study, differentiated culture model of human corneal epithelium was developed as a tool for drug and gene delivery studies. The culture conditions, morphology and physical barrier properties of the cultured epithelium were evaluated. The usefulness of the new cell model was also tested in gene delivery experiments with non-viral vectors.

6.1 Culture conditions

Differentiation process of corneal epithelial cells *in vitro* is a combination of soluble inducers in culture medium, used permeable support system and cell grown on air-lift.

The feeding medium of the HCE-cells includes 15% of FBS. Serum represents most of the factors required for cell proliferation and maintenance and in addition buffers the cell culture system against a variety of perturbations and toxic effects (Cartwright and Shah 2002). Disadvantages in the use of serum are lack of reproducibility which leads to different absolute and relative levels of growth factors, protein and monoclonal antibody existence. High concentrations of serum have been noticed to disturb cell proliferation and differentiation (Ahmed and Patton 1985; Kruse and Tseng 1993). Some groups have reported improved differentiation of the corneal epithelium in serum free medium (Kruszewski et al., 1997; Castro-Muñozledo et al., 1997) and many immortalised corneal epithelial cell lines are grown in serum-free medium (Offord et al., 1999; Mohan et al., 2003a; Robertson et al., 2005). HCE-cells were also cultivated at lower serum concentrations (2%, 5%, 10%), but in these conditions the cells did not survive. On the other hand, any extra supplements e.g. growth factors were not added in culture medium to compensate for reduced serum concentration. Furthermore, the differentiation of corneal epithelial cells growing in serum-free medium is stimulated with changes in ionic calcium concentrations (Ward et al., 1997; Mohan et al., 2003a;

Robertson et al., 2005). In our studies, Ca^{2+} concentration remained stable while HCE-cells grew at reduced serum concentration. HCE-cells have also been grown successfully in serum free medium with or without animal based extracts (Wilkinson and Clothier 2005). The growth was equal in the both methods, but ZO-1 localization was seen only at Ca^{2+} concentration of 100 μM or above. However, the cells were investigated only as monolayers.

Filter material with its pore size and the coating components was seen to be important factors in HCE-cell differentiation. Polyester filters with 0.4 μm pore size proved to be better than polycarbonate filters for barrier formation with HCE-cells, and the clear polyester membrane also provides better cell visibility using light microscopy or phase-contrast microscopy.

Human corneal epithelium cultured on collagen gels can synthesize and deposit basement membrane components like laminin and type IV collagen (Ohji et al., 1994; Fukuda et al., 1999). HCE-cells have been proved to express laminin and two fibronectin isoforms (Ebihara et al., 2000; Filenius et al., 2001 and 2003). The mixture of collagen and laminin represents the basal lamina in HCE-model and further improves the differentiation features of the model. However, the cells did not differentiate when growing on commercially available basement membrane matrix (MATRIGEL®) (unpublished data), which includes laminin, collagen and some growth factors. Collagen with mouse 3T3-fibroblast resembles a corneal stroma in HCE-model and interactions between the fibroblasts and corneal epithelial cells stimulate the differentiation of the HCE-cells. Based on morphology and on the barrier properties, a polyester filter coated with laminin/collagen, collagen or collagen/fibroblasts mixture appeared to be optimal for HCE-cell differentiation. In other corneal epithelial models, the cells have grown both on polyester and polycarbonate filters coating with collagen type I, laminin or fibronectin (Table 5, p. 45). Mixture of collagen and fibroblasts has been mainly used in the organotypic whole cornea models.

The air-liquid interface seems to be the most critical for the differentiation of HCE-cells as has previously been observed in other corneal epithelial cell models (Table 5). No flat apical cells or proper barrier was obtained without air lifting in HCE-culture. The apical surface of the corneal epithelium contributes about 70% of the total electrical

resistance of the cornea (Klyce 1972) and the top two layers are the most important part of the cornea in limiting the permeability of hydrophilic drugs (Klyce and Crosson 1985). HCE-model shows tight junctions and desmosomes in the flattened apical cell layer, but the wing cells and basal cells in the cultured epithelium are not organized as well as they are in the intact cornea. The HCE-model does not include stroma, only 'stroma-like' much thinner 'extracellular matrix'. Stroma and endothelium are not critical barriers in the corneal drug absorption (Prausnitz and Noonan 1998).

In optimal conditions, the TER values of the HCE-model were 400 – 1200 Ωxcm^2 which is at lower level than the TER-values of intact cornea. TER-values of the differentiated HCE-cells are in line with other human corneal epithelial cell models (Table 5). In addition, TER-values of HCE-T cells grown without airlift remained ~200 Ωxcm^2 (Ward et al., 1997) that was seen also in HCE-cell grown. No flat apical cells or proper barrier was obtained without air lifting in HCE-culture and TER-values lower than 400 Ωxcm^2 with poor hydrophilic drug permeation indicated failed terminal cell differentiation.

6.2 Paracellular and transcellular permeability

Paracellular permeation takes place via the spaces between the cells. These spaces are limited by the tight junctions in the cornea epithelium (Marshall and Klyce, 1983). Major determinants of the paracellular permeability are the dimensions of the paracellular space and permeating molecule.

HCE-cells have slightly larger paracellular pores than the excised cornea, but the pore density is less than in the rabbit cornea (**II**; Fig. 2, Table 1). Due to the larger intercellular space (i.e. higher porosity) the HCE-model shows 2 – 4 times higher or similar permeability for the hydrophilic compounds (6-carboxyfluorescein, mannitol, PEGs, hydrophilic β-blockers), (**I**; Table 1, **II**; Fig. 1 and 3, Table 2). However, these differences are relatively small. Furthermore, the paracellular permeability in rabbit cornea may be less than in the human cornea (Urtti and Salminen, 1993). This has also been seen in this study, as the paracellular space of the HCE-model is slightly wider than in the rabbit cornea. Paracellular dimensions of any other corneal cell culture model have not been determined before.

Table 5. Cell culture models of the corneal epithelium (revised from Hornof et al., 2005).

Species	Cell culture conditions	TER [Ω·cm^2]	Characterisation	Applications	Ref.
Primary cells					
rabbit (RCEC)	collagen-coated membrane; medium contains 5% serum; culture time 8 days	~ 100-150	morphology; bioelectric parameters; permeability	permeability and active transport studies	[1-3]
rabbit (RCrECL)	fibronectin / collagen / laminin-coated membrane; serum-free medium; air-lifted; culture time 7-8 days	~ 5000	morphology; bioelectric parameters; keratin expression; permeability	permeability studies	[4-6]
rabbit	collagen coated membrane; medium contains 10% serum	~200	morphology; bioelectric parameters;	toxicity	[7]
Immortalised cells					
human (HCE-T 10.014)	collagen-coated membranes; serum-free medium; air-lifted; culture time 6 days	~ 400-600	morphology; bioelectric parameters; karyotype; isozyme; keratin expression	cell biology; toxicity; ocular irritancy; gene regulation studies	[8-10]
human (HCE)	collagen-coated/ fibroblasts membrane; medium contain 15% serum; air-lifted; culture time 3-4 weeks	~ 400-800	morphology; bioelectric parameters; permeability; esterase activity; paracellular pore size and density	permeability studies; prediction of ocular pharmacokinetics by combining in vitro permeation data with computer simulation program	[11]
human (HPV 16-E6/E7)	collagen-coated membrane; EpiLife medium; air-lifted; culture time 4-12 days	~ 400-500	morphology; bioelectric parameters; cytogenetics	toxicity	[12]
rabbit (RRCE)	polyester membrane; medium contains 15% serum; air-lifted; culture time 2-6 weeks	~ 150	morphology; bioelectric parameters; permeability	permeability studies	[13]

[1-3] Kawazu et al., 1998, 1999 a and b, 2006; [4] Chang et al., 2000; [5] Scholz et al., 2002; [6] Chang-Lin et al., 2005; [7] Wang et al., 2001; [8] Kahn et al., 1993; [9] Ward et al., 1997; [10] Kruszewski et al., 1997; [11] Ranta et al., 2003; [12] Mohan et al., 2003a; [13] Burgalassi et al., 2004

In other models, permeabilities of hydrophilic markers in primary corneal epithelial cell model are practically the same as in intact rabbit cornea (Chang et al., 2000), but Kawazu's et al. (1998) model showed levels of permeability about 100 times higher permeabilities than those of the whole cornea. Permeation of sodium fluorescein in HCE-T and HPV16-E6/E7 models was studied by Ward et al. (1997) and Mohan et al. (2003a). After a 30-min exposure, HCE-T and HPV16-E6/E7 cultures allowed permeation of about 5% and 9% of the sodium fluorescein as compared to filter control, respectively. Since only one time point was used, P_{app} in cm/s cannot be evaluated reliably.

In general, increased lipophilicity facilitates the corneal permeability. Lipophilic transcellular marker rhodamine B exhibited a high permeability across the HCE-cell culture and the value was practically the same as in the isolated rabbit cornea (**I**; Table 1). Permeability studies of β-blockers across differentiated HCE-cells shows a parabolic relationship between the lipophilicity (i.e. logP) and permeability (**II**; Fig. 3). Similar relationship has been shown for isolated cornea with β-blockers and pilocarpine prodrugs (Huang and Schoenwald 1983; Wang et al., 1991; Suhonen et al., 1991 and 1996) and primary rabbit corneal cell culture model (Kawazu et al., 1998), but in this case the levels of permeability were at much higher level than in other studies.

Due to the transcellular permeation with increasing lipophilicity (logP > 1.7) the permeability of β-blockers in the HCE-model and in the excised cornea improved substantially. At high lipophilicity (logP > 2.5), the P_{app} in the HCE-model was less than in the excised cornea. Highly lipophilic drugs penetrate easily to the lipophilic corneal epithelial cells, but their transfer into the hydrophilic stroma becomes the rate-limiting step (Schoenwald and Huang 1983). The properties of the corneal stroma and the collagen matrix and filter of the HCE-model differ from each other by thickness, construction and permeability features. The experiments showed that the permeability in the filter is independent on lipophilicity and the rate-limiting factor shifts from the HCE-cells to the filter and matrix, with increasing lipophilicity. The permeability coefficients of hydrophilic and lipophilic β-blockers in the corneal stroma, ~ (32 – 35) x 10^{-6} cm/s (Huang et al., 1983), were higher than in the coated filters in the present

study, (23 – 24) x 10^{-6} cm/s. This is in line with the lower permeabilities of lipophilic drugs in the HCE-model.

The degree of ionization is the third important factor which affects the corneal penetration so that unionized drug usually permeates the epithelium more easily than its ionized form (Ramer and Gasset 1975; Sieg and Robinson 1977; Mitra and Mikkelson 1988; Suhonen et al., 1998). The better permeability of unionized drugs is based on their higher lipophilicity unless the lipophilicity is very high (Suhonen et al., 1998). Thus, transcellular permeability increases with increasing fraction of the unionized drug.

At physiological pH or pH above the pI (3.2) of paracellular protein, the corneal epithelium is negatively charged and paracellular permeability is selective to positively charged solutes (Rojanasakul et al., 1992). At pH values below the pI, the reverse order is observed. It has been shown in previous studies that protonated hydrophilic amines permeated e.g Caco-2 epithelial cell monolayers faster than their neutral forms (Adson et al., 1994), whereas hydrophilic peptides at positive net charge permeate Caco-2 cell monolayers slower than neutral compounds (Pauletti et al., 1997). The levels of permeability of positively charged PEGs across HCE-model were mainly at a level 2-fold lower than those of neutral PEGs at the similar molecular weights. Positively charged PEGs may bind to negatively charged functional groups in the cell membrane too tightly which may decrease the paracellular permeability of these compounds. Furthermore, pH might affect uncharged PEGs in water at different pH values.

Any evidence of P-gp activity was not seen in propranol permeability across HCE-cell layers (**II**; Table 2), although propranolol (Hamilton et al., 2001) as well as talinolol (Hilgendorf et al., 2000) are P-gp-substrates.

The permeation studies of new ocular drug candidates have traditionally been performed using excised rabbit corneas. However, this method does not take into account the limited contact time of the solution on the ocular surface *in vivo* or the rate of drug desorption from the cornea into the receiver solution. Ranta et al. (2003) determined the absorption and desorption rates of β-blockers in the HCE model. These data and relevant pre-ocular and intraocular kinetic parameters were incorporated into ocular pharmacokinetic model to simulate the *in vivo* situation. The simulated timolol

concentration profiles in the rabbit aqueous humor were similar, but not identical, with *in vivo* studies.

6.3. Esterase activity

The corneal epithelium is known to be metabolically active, which may limit permeation of many drugs (Lee 1983; Harris et al., 1992), but also many ophthalmic drugs are applied as ester prodrugs to achieve a higher bioavailability. However, determination of esterase activity is rarely used in characterisation of corneal cells. Previously permeation and metabolism studies by Meyer et al. (2005) indicated that the HCC-whole cornea model is able to adequately convert hydrocortisone acetate to hydrocortisone.

Permeability study with an esterase substrate fluorescein diacetate showed that only fluorescein was found in the basolateral side of the HCE-model. The improvement in permeability (i.e fluorescein and fluorescein diacetate) in the HCE-model was 49-fold and in the rabbit cornea 31-fold. These results show that the HCE-model may be applicable to prodrug studies. However, many types of esterases with different activities as well as peptidases and proteases exist, and more detailed characterisation of metabolic activity in HCE-cells has not been determined.

6.4 Gene transfer

Despite poor bioavailability topical delivery is the most common route of ocular drug delivery. However, topical application is an ineffective delivery system in the use of hydrophilic and protein drugs of larger size. Moreover, use of many protein drugs is limited by their manufacturing cost, chemical and biological instability. Administration through systemic circulation is also inefficient because of blood-retinal barrier and/or rapid hepatic metabolism and renal excretion. Furthermore, very high doses of protein drugs for compensation of low permeability may lead to toxic side effects (Banga 1996).

According to the present study, SEAP protein concentration in eye drops should be 13% to reach steady-state concentration in the aqueous humor. Such high concentration

is impossible for a protein drug. Gene transfer of corneal epithelium may offer an effective possibility to overcome the tight barrier of corneal epithelium.

Reporter gene- Recombinant alkaline phosphatases are efficiently secreted from transfected cells. SEAP can be simply studied by repeated collection of the culture medium from the same cultures. SEAP concentration in culture medium is directly proportional to changes in intracellular SEAP mRNA (Berger et al., 1988). So far, there are only a few reports about transfection of SEAP-gene into eye cells *in vitro* (Maruyama et al., 2001 and 2002; Mannermaa et al., 2005) and none *in vivo*. Pharmacokinetic studies of transferred protein expression in the corneal epithelium using SEAP as a reporter gene *in vitro* and *in vivo* are not available.

Secretion of reporter gene- Secretion rate of SEAP was 2.6 times greater into aqueous humor than into tear fluid. SEAP found in aqueous humor comes from cornea, but the proportions of SEAP secretion from cornea and conjunctiva into tear fluid are not known. However, real-time RT-PCR results suggest a greater rate of secretion from the conjunctiva (**III**; Fig. 6).

Secretion rate of SEAP to the basolateral side of differentiated HCE-cells was only 2.6-fold greater than the *in vivo* rate into aqueous humor during the three days after transfection. Influence of exposure time of DNA complexes on transfection was not studied, but shorter periods *in vitro* probably would lead to lower expression levels. The data analysis shows that the differentiated HCE-model secretes transgene product SEAP approximately at the same rate corneal epithelium does *in vivo* suggesting that the cell model gives realistic prediction. Transfection levels decreased with the cell differentiation (**III**; Fig. 7): cumulative basolateral secreted SEAP from differentiated cells in three days was 250 times less than from the dividing HCE-cells demonstrating the importance of differentiated cell model.

Transfection efficiency- The relative transfection efficacy of different carriers was dependent on the stage of differentiation. PEI was the most effective gene carrier to dividing/differentiating cells, but it did not transfect differentiated HCE-cells. The ability of PEI to transfect dividing cells compared to other carriers like DOTAP/DOPE has been shown also in some other cells (Ruponen et al., 2001; Männistö et al., 2005; Reinisalo et al., 2006). In differentiated and polarized retinal pigment epithelial cell line

(RPE), the high cellular uptake of PEI complexes did not lead to transfection (Mannermaa et al., 2005). DOTAP/DOPE complexes mediated higher transfection in the differentiated HCE-model than PEI although PEI was more effective in dividing/differentiating cells. In addition, transferrin-PEI conjugate was effective in the transfection of rabbit corneal endothelial cells *in vitro* but not *ex vivo* (Nguyen et al., 2002).

PSs, which are naturally occurring substances found only in sperm, has been shown to condense pDNA efficiently for delivery into several different types of cells *in vitro* by several different types of cationic liposomes (Sorgi et al., 1997). In previous studies, the transfected gene expression has been shown to be higher with liposomal/PS complexes than liposome complexes alone (Pleyer et al., 2001; Mannermaa et al., 2005). However, PS did not improve the efficiency of gene transfer with DOTAP/DOPE in HCE-model.

Administration- Despite the use of a transfection vector the administration of transferred genes into cornea has usually been performed by injections *in vivo* (Table 3, p. 28; Table 4, p. 32). Corneas have also been transfected *ex vivo* and then transplanted *in vivo* (Larkin et al., 1996; Rayner et al., 2001). Lately the ocular tissues have successfully transfected via intravenous administration using non-viral vectors with gene-targeted technology and tissue-specific gene promoters (Zhu et al., 2002; Zhang et al., 2003). Topical administration is quite rarely used in gene transfer, because of rapid elimination of eye drops from precorneal area and epithelial tight barrier. In some studies, the epithelial superficial cells have been scarified before topical administration of foreign genes. Masuda et al. (1996) and Matsuo et al. (1996) reported that topical application of liposome eye drops into rat eyes could transfer gene to corneal epithelium and even to the retinal ganglion cells, but these results have not been supported later by other studies.

Higher viscosity and longer contact time of eye drops on the cornea may increase transfection levels (Adler et al., 1971; Patton and Robinson 1976; Chastain 2003), if formulation technologies can be developed for that purpose. Furthermore, conventional methods like to decrease the drainage loss by adjusting instillation volume and time interval between drops (Chrai and Robinson 1974), closure of the eyelids (Zimmerman

et al., 1984) or blocking the nasolacrimal duct (Kaila et al., 1986) may facilitate complex delivery into the cornea. Transfection efficiency may also be improved by vector design such as the use of tissue specific, strong or inducible promoters, replicating plasmids and gene vector targeting to cell surface receptors (Hart et al., 1998; Borrás 2003; Tan et al., 2006).

7 CONCLUSIONS

In the present study the *in vitro* cell model of human corneal epithelium (HCE-model) was developed and characterised for ocular drug studies. The main conclusion is that the model is a useful and realistic model in corneal permeation and gene transfer studies. The specific conclusions of the study are the following:

1. The HCE-model resembles intact corneal epithelium with morphologically identifiable desmosomes, tight junctions, microvilli, and cell layers with apical flat cells.

2. The passive permeabilities of hydrophilic and lipophilic model compounds across the HCE-model correlate well with the intact rabbit cornea. The differences in permeabilities between HCE-model and rabbit cornea were practically less than 2-fold. Paracellular permeability was slightly higher whereas transcellular permeability lower than that of excised rabbit cornea. HCE model can be used to predict ocular absorption of the drugs that permeate through the cornea by passive diffusion. However, the expression of the active transporters, efflux proteins, metabolic enzymes, surface proteins and mucins in the differentiated HCE-cells are so far unknown.

3. HCE-model proved to be a promising method to screen chemical gene delivery vectors. Importance of differentiation is demonstrated both in terms of SEAP secretion levels and by the rank order of delivery systems. The transgene expression levels strongly decreased with the increased differentiation of corneal epithelial cell. Accordingly, it was proved that functional delivery system *in vitro* in proliferating cells may not function *in vivo*. In differentiated cells the rate of SEAP secretion showed similarity with *in vivo* results.

4. Prolonged protein secretion into the tear fluid can be obtained by transfecting the corneal epithelium which serves as secretion platform of protein.

5. Therapeutic protein can be delivered into the anterior eye chamber by topical transfection of the corneal epithelium, which then secretes the protein to the basolateral side and further to the anterior chamber. As such proteins can not penetrate from the tear side across the corneal epithelium.

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