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Review article

Cell culture models of the ocular barriers ☆

Margit Hornof, Elisa Toropainen, Arto Urtti*

Department of Pharmaceutics, University of Kuopio, Kuopio, Finland

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Abstract

The presence of tight barriers, which regulate the environment of ocular tissues in the anterior and posterior part of the eye, is essential for normal visual function. The development of strategies to overcome these barriers for the targeted ocular delivery of drugs, e.g. to the retina, remains a major challenge. During the last years numerous cell culture models of the ocular barriers (cornea, conjunctiva, blood–retinal barrier) have been established. They are considered to be promising tools for studying the drug transport into ocular tissues, and for numerous other purposes, such as the investigation of pathological ocular conditions, and the toxicological screening of compounds as alternative to in vivo toxicity tests. The further development of these in vitro models will require more detailed investigations of the barrier properties of both the cell culture models and the in vivo ocular barriers. It is the aim of this review to describe the current status in the development of cell culture models of the ocular barriers, and to discuss the applicability of these models in pharmaceutical research.

Keywords: Cell culture model; Blood–retinal barrier; Blood–aqueous barrier; Cornea; Conjunctiva; Retinal pigment epithelium; Retinal capillary endothelium; Ocular drug delivery; Ocular absorption

1. Introduction

In order to function as organ of vision it is of vital importance for the eye to maintain a highly regulated environment for the visual cells and transparent tissues. Consequently, tight cellular barriers, which restrict and regulate the uptake of fluids and solutes, are present in the anterior and posterior parts of the eye. These barriers are fundamentally important for the protection of the eye and for the maintenance of vital ocular functions.

Ocular barriers effectively protect the eye also from pharmaceuticals. The development of strategies to overcome these barriers for the targeted ocular delivery of drugs remains a major challenge for pharmaceutical scientists. Especially as new therapeutic strategies for the treatment of ocular diseases become available (e.g. new drugs, proteins, gene medicines), methods to increase the bioavailability of ocularly applied drugs and, in particular, the delivery of drugs to the retina are the subject of extensive research.

Animal experimentation is an essential part in the research and development of ocular drugs and delivery systems. The applications for animal experiments include pharmacokinetic and pharmacodynamic studies and toxicity evaluations (e.g. Draize test). The rabbit is the most commonly used animal model. Larger animals, like pigs, dogs, cats, and monkeys are rarely used, and the small eye size of mice and rats limits their value in ocular studies. However, there are morphologic and biochemical differences between the rabbit eye and the human eye. The most obvious disadvantage of rabbits as animal model for ocular pharmacokinetic studies is their infrequent blinking rate. The low blinking frequency of about $5\,h^{-1}$ decreases precorneal drainage of topically applied solutions in comparison to humans with a blinking frequency of 6–7 min^{-1} [1]. Ocular pharmacokinetics are also influenced by the 1.5- to 2-times larger corneal surface area of rabbits. In addition, the conjunctival surface area of the rabbit is 9 times larger than its corneal surface area. In humans the difference is 17-fold [1]. As a consequence, the ocular bioavailability of topically applied drugs in the rabbit is less influenced by the non-productive absorption through the conjunctiva. Another difference between human and rabbit

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* Corresponding author. Department of Pharmaceutics, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland. Tel.: +358 17 162480; fax: +358 17 162252.
E-mail address: arto.urtti@uku.fi (A. Urtti).

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eye is the absence of melanin pigment in the anterior uvea of albino rabbits. In humans (and pigmented rabbits) many drugs bind to the melanin pigmentation of the anterior uvea, which retards their elimination from the anterior chamber and prolongs their activity [1,2].

Animal experiments have been widely criticized for ethical and economical reasons. Rabbits have to be sacrificed at each time-point to collect tissue samples in ocular pharmacokinetic studies. For example, in order to compare the pharmacokinetic profiles of a drug in two different delivery systems using six animals for each delivery system and measuring 8 time points, either 48 or 96 rabbits have to be sacrificed, depending on whether one or both eyes are used for the study. Within the European Union the protection of animals used for experimental and other scientific purposes is covered by Directive 86/609/EEC, which aims at reducing the numbers of animals used for experiments. It is required that an animal experiment should not be performed when an alternative method exists [3]. Consequently, the development and validation of new in vitro tests to replace animal experimentation in Life Sciences is among the priorities of the current 6th European Community framework program for research, technological development and demonstration.

Cell culture models of the ocular barriers provide powerful systems for the pharmaceutical scientist to investigate the architecture, barrier function and regulation of the ocular barriers in vitro. Furthermore, cell culture models can be used for numerous other purposes such as studying passive and active transport of drugs and endogenous substances, cell physiology, metabolism, and protein expression, for the development of delivery systems for genes and antisense oligonucleotides, and for in vitro toxicity tests. These models can also be used to identify compounds and formulations with favourable pharmacokinetic properties and to evaluate structure–absorption and structure–metabolism relationships.

Cell culture models offer the advantage of a highly defined system, in which parameters and conditions can be easily changed. The results are often more reproducible in comparison to in vitro studies with excised animal tissue. Moreover, the use of human cell lines avoids the species related applicability problems that might arise when using animal tissue for in vitro experiments.

The use of both primary and secondary cell culture systems for modelling the ocular barriers is described in the literature. Immortalized human cell lines derived from corneal epithelial [4–8], conjunctival epithelial [8,9] and retinal pigment epithelial cells [10,11] are currently available. Immortalized cell lines have some advantages over primary cells. Especially, the isolation of tissues, followed by harvesting and purification of cells, is not required. In addition, the characteristics of the cultures remain stable for a large number of passages, and cell cultures can be rapidly expanded if a large number of cells is needed for experiments [12]. On the other hand, after immortalization these cell lines may have abnormal characteristics compared to primary cells, and their gene expression profile might be significantly different from the normal, native cells [13]. If adequate conditions are used, the cells in primary culture are likely to reflect in vivo cell morphology and function more accurately. These cells, however, stop growing after a few passages in culture and they revive after storage in liquid nitrogen weakly or not at all. Therefore, new cells have to be isolated frequently, and taking the limited availability of human donor eyes into account, it becomes clear that the primary cell culture methods are not optimal for larger scale screening of pharmaceutical compounds and formulations.

It is the aim of this review to describe the current status in the development of cell culture models for the ocular barriers, and to discuss the applicability of these models in pharmaceutical research.

2. General view on ocular barriers and pharmacokinetics in the eye

2.1. Tear fluid–eye barriers

Most ocular drugs are applied locally as eyedrops. However, due to lacrimal drainage and the systemic absorption in the conjunctiva, only a small fraction of the drug is absorbed into the eye [14,15]. After the topical administration of a drug to the ocular surface, there are two main pathways of drug entry into the anterior chamber: via the cornea and via the conjunctiva (Fig. 1), the latter route being of minor relevance for most drugs [2,15]. Small and lipophilic molecules (i.e. most clinically used drugs) are absorbed via the cornea, whereas large and hydrophilic molecules (e.g. new potential biotech-drugs such as protein and peptide drugs, gene medicines) are preferably absorbed through the conjunctiva and sclera [16,17].

2.1.1. Cornea

The cornea is an important mechanical and chemical barrier, which limits the access of exogenous substances into the eye and protects the intraocular tissues. The cornea is a clear and avascular structure with average diameter and thickness of 12 mm and 520 μm, respectively [2]. The human cornea consists of five layers: corneal epithelium, basement membrane, Bowman's layer, stroma, Descemet's membrane and endothelium (Fig. 2) [18].

The corneal epithelium is composed of two to three cell layers of flattened superficial cells, two to three cell layers of wing cells, and a single layer of columnar basal cells. The superficial cells adhere to one another via desmosomes and the cells are encircled by tight junctions [19]. Due to these tight junctions the corneal epithelium represents the rate-limiting barrier for the permeation of hydrophilic drugs, whereas the stroma and endothelium offer very little

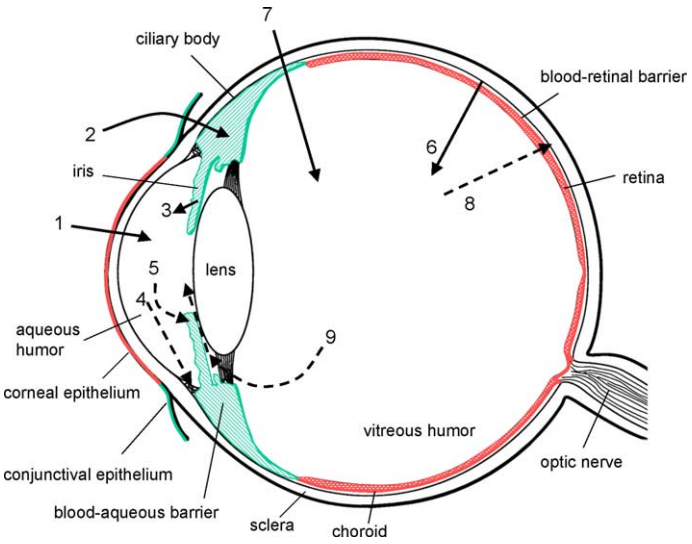

Fig. 1. Biological barriers of the eye (tight barriers are indicated in red, others in green; ---> route of elimination). The main pathway for drugs to enter the anterior chamber is via the cornea (1). Some large and hydrophilic drugs prefer the conjunctival and scleral route, and then diffuse into the 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).

resistance to transcorneal permeation [20,21]. The stroma, which constitutes 85–90% of the total corneal mass, is mainly composed of hydrated collagen. It exerts a diffusional barrier only to highly lipophilic drugs due to its hydrophilic nature [20]. The corneal endothelial monolayer maintains an effective barrier between the stroma and aqueous humor. Active ion and fluid transport mechanisms in the endothelium are responsible for maintaining corneal transparency [22].

The passive permeability of a drug across the cornea is influenced by various factors, such as the lipophilicity, molecular weight, charge, and degree of ionization of the drug [17]. It was shown that increasing molecular size of the permeating substance decreases the rate of paracellular permeation [23]. The pore size in the intercellular space is an important factor influencing paracellular permeation of drugs. Only relatively small molecules can permeate through the pores, which have an average diameter of 2.0 nm in the rabbit [23]. As the pores of the corneal epithelium are negatively charged at physiological pH, negatively charged molecules permeate slower than positively charged and neutral molecules [24]. Positive charge may decrease the permeation in some cases as well (e.g. cationic aminoPEGs) [25]. This is probably due to ionic interaction between the positively charged molecules and the negatively charged carboxylic groups of tight junction proteins.

Lipophilic drugs permeate faster and to a greater extent through the cornea by the transcellular way, the main route of ocular absorption for clinically used drugs. For example, the permeability coefficient (P_{app}) of the lipophilic beta-blocker betaxolol (log D 1.59 (pH 7.4); P_{app} 2.7×10^{-5} cm/s) was 25

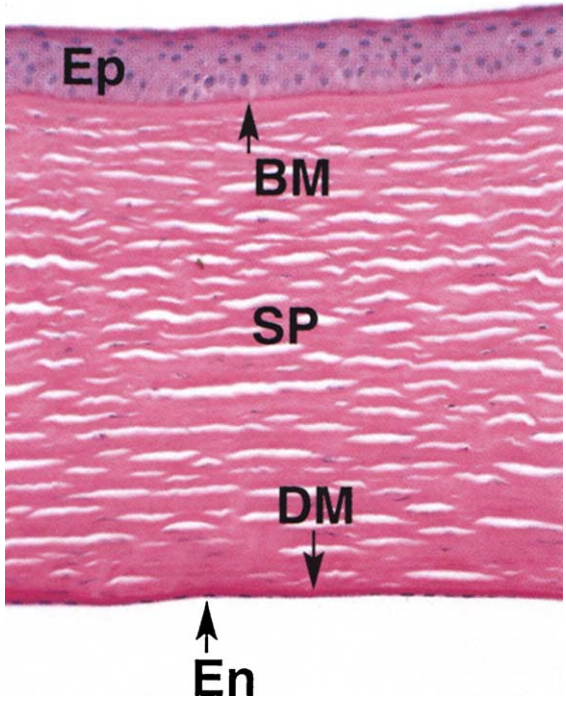

Fig. 2. Microphotograph of human cornea (haematoxylin and eosin staining, magnification ×100). The outermost layer of the cornea is the epithelium Ep, which rests on a basement membrane supported by a specialized layer of corneal stroma called Bowman's layer (membrane) BM. The corneal stroma (substantia propria) SP consists mainly of collagen lamellae. The inner surface of the cornea is formed by endothelial cells En, which are supported by Descemet's membrane DM. Reprinted from Wheater's Functional Histology [18] with permission of Harcourt Publishers Ltd.

times higher than the permeability coefficient of the hydrophilic atenolol with similar molecular size (log D −1.77 (pH 7.4); P_{app} 1.1×10^{-6} cm/s) in the isolated rabbit cornea [26,27].

After crossing the cornea the drug diffuses into the aqueous humor and to the anterior uvea. Locally applied drugs that enter the eye via the corneal route cannot reach the retina and vitreous at sufficient therapeutic concentrations [28]. For instance, 30 min after eyedrop administration the concentration of timolol in the vitreous humor was about 26 times lower than the concentration in the aqueous humor [29].

2.1.2. Conjunctiva

The conjunctiva is a mucous membrane consisting of an epithelium, which is two to three cell layers thick, and an underlying vascularised connective tissue. It covers the anterior surface of the sclera (bulbar conjunctiva), and is folded at the fornix (fornix conjunctiva) to form the palpebral conjunctiva, which lines the inner surfaces of the eyelids. The conjunctival epithelium plays an important role as protective barrier on the ocular surface, and it contributes to the maintenance of the tear film by the production of mucus glycoproteins. On the apical surface of the epithelium tight junctions are present. The bulbar conjunctiva represents the first barrier for the permeation of topically applied drugs into the eye via the non-corneal route. This route of intraocular entry is relevant for large and hydrophilic substances, which are poorly absorbed through the cornea [16,30]. It was shown in the rabbit that the conjunctival epithelium has 2 times larger pores and a 16 times higher pore density than the corneal epithelium, which results in a 15- to 25-fold higher permeability in comparison to the cornea [23].

A significant portion of the drug is lost to the systemic circulation while crossing the conjunctiva (Fig. 3) [31–33]. This non-productive absorption is the main reason for the poor bioavailability of drugs entering the eye via the conjunctival/scleral pathway [30]. The remaining drug can diffuse through the sclera, which consists mainly of collagen and mucopolysaccharides, and, in contrast to the conjunctiva, is only poorly vascularised. The sclera is about 10 times more permeable than the cornea and half as permeable as the conjunctiva [23]. The drug can enter the posterior part of the eye (uveal tract, retina, choroid, vitreous humor) using this route [30].

2.2. Blood–ocular barriers

The establishment and maintenance of therapeutically relevant drug concentrations in the retina and the vitreous usually require either systemic or intravitreal drug administration. Systemic application by oral or intravenous administration has the disadvantage that high doses of the drug have to be administered, since only a very small fraction of the drug reaches ocular tissues due to the limited blood flow and the blood–ocular barriers. The remainder of the drug is distributed in the entire body leading to unwanted side effects. The blood–ocular barrier can be overcome by the intravitreal injection of drugs (Figs. 1 and 3), but this route of administration is associated with several problems, including risks of endophthalmitis, damage to lens or retinal detachment, and low patient compliance [34].

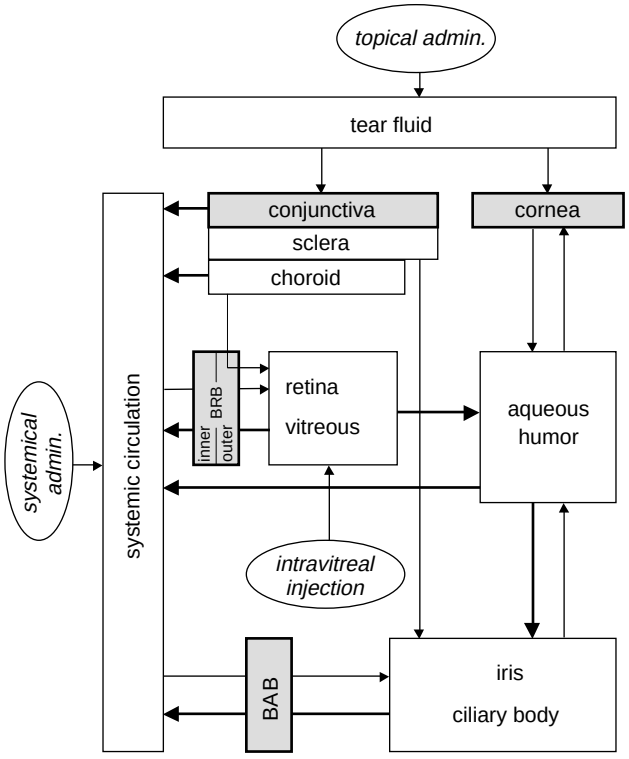

Fig. 3. Ocular penetration routes for drugs after topical, systemical and intravitreal administration. The main elimination pathways are indicated by bold arrows; the ocular barriers (BRB, blood–retinal barrier; BAB, blood–aqueous barrier) are indicated by a grey background.

There are two blood–ocular barrier systems in the eye: the blood–aqueous barrier and the blood–retinal barrier (Fig. 1), which provide a controlled environment for the internal ocular tissues.

2.2.1. Blood–aqueous barrier

The blood–aqueous barrier (BAB) is located in the anterior part of the eye (Fig. 1). It is formed by endothelial cells of the blood vessels in the iris, and the non-pigmented cell layer of the ciliary epithelium. Tight junctional complexes are present in both cell layers. These cell layers prevent non-specific passage of solutes into the intraocular milieu, that might otherwise negatively influence transparency and chemical equilibrium of the ocular fluids [35]. However, even when the BAB is intact, its barrier function is not complete. For instance, whereas injected horse radish peroxidase (HRP, molecular mass 40 kDa) cannot pass through the iris blood vessels, it can permeate through the fenestrated capillaries of the ciliary processes, and reach the aqueous humor [36,37]. This permeability accounts for the presence of low levels of plasma proteins in the aqueous humor.

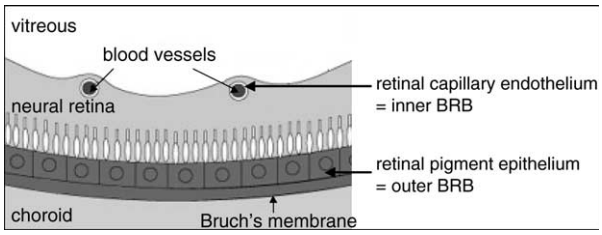

Fig. 4. Schematic presentation of the blood–retinal barrier (BRB). Both the inner and the outer BRB hinder drug transport from blood vessels to the neural retina.

The outward movement of substances from the aqueous humor across the iris blood vessels into systemic circulation is less restricted [38]. The iris tissue itself is porous, and drugs dissolved in the aqueous humor can freely enter across its anterior surface. Some drugs are then absorbed by the iris pigments or they are removed from the anterior chamber by passage into the iris blood vessels [2,15]. Especially, small and lipophilic drugs can enter the uveal blood circulation via the blood–aqueous barrier and they are consequently eliminated more rapidly from the anterior chamber than larger and more hydrophilic drugs, which are eliminated by aqueous humor turnover only [1]. For instance, the clearance of pilocarpine was determined to be 13.0 μl/min, whereas the clearance of inulin was close to the rate of aqueous humor turnover [39,40]. Although aqueous humor turnover, which is 3.0–4.7 μl/min in rabbits and 2.0–3.0 μl/min in humans [1,39,41], is often considered to be the major route of drug elimination from the anterior chamber, many drugs have clearance values exceeding the rate of aqueous humor turnover, which indicates the presence of alternative elimination pathways [1,40].

2.2.2. Blood–retinal barrier

The blood–retinal barrier (BRB) is located in the posterior part of the eye. It is formed by the endothelial cells of retinal blood vessels (inner blood–retinal barrier) and the retinal pigment epithelial cells (outer blood–retinal barrier) (Fig. 4).

The multilayered neural retina is separated by the subretinal space from the retinal pigment epithelium (RPE) monolayer, which separates the outer surface of the neural retina from the choroid (Fig. 5). Under physiologic conditions the retina is firmly attached to the RPE. The RPE plays a vital role in maintaining the viability and function of the neural retina [42]. The RPE is, for example, responsible for the removal of fluid from the subretinal space in order to maintain retinal adhesion and to keep the neural retina in a state of dehydration. Because of its tight junctions the RPE forms a strong barrier, but it is capable of a number of specialized transport processes. Only selected nutrients are exchanged between choroid and retina, the transcellular and paracellular passage of other molecules across the RPE is restricted [28]. Because of their specialized functions the RPE cells have unique morphologic and functional polarity properties. One feature of RPE cells is the predominant apical localization of Na^+,K^+-ATPase, which regulates intracellular Na^+ and K^+ homeostasis [43].

Apart from forming the blood–retinal barrier, the RPE has several other functions, such as the phagocytosis of the outer segments shed by retinal rods and cones; transport of nutrients and metabolic products; retinoid transport and metabolism; the absorption of light and the dissipation of heat energy derived from incident light; synthesis of enzymes, growth factors and pigments; and participation in the immune reactivity of the retina [42].

The inner blood–retinal barrier is constituted by endothelial cells, which cover the lumen of retinal capillaries and separate the retinal tissue and the blood. The retinal endothelial cells have intercellular tight junctions. Glial cells (astrocytes and Müller cells) are in contact with the vessel walls and provide signals which induce barrier properties in the endothelial cells [44]. Due to these tight junctions the paracellular transport of compounds is restricted [35]. In these respects the inner blood–retinal

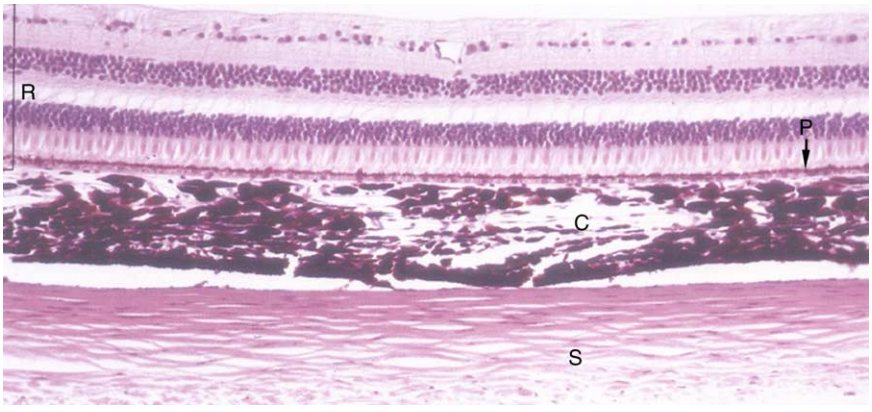

Fig. 5. Microphotograph of the wall of the eye (haematoxylin and eosin staining, magnification ×300). The wall of the eye consists of three layers. The innermost layer is the retina R, which is separated by Bruch's membrane from the choroid C. The choroid C is a vascular supporting tissue between retina R and sclera S. The outer blood–retinal barrier is constituted by the outermost layer of the retina, the retinal pigment epithelium P. Reprinted from Wheater's Functional Histology [18] with permission of Harcourt Publishers Ltd.

barrier is similar to the blood–brain barrier, which is constituted by the endothelial cells of the microvessels in the brain, pericytes and astrocytes. While the density of tight junctions and cytoplasmic vesicles was determined to be higher in retinal vessels than in the brain, pericytes are 4 times as numerous in the retina, which probably compensates for the more permeable endothelial barrier [45]. Stewart and Tuor determined with a vascular tracer that transfer by passive diffusion is about 4 times higher in the retina than in the brain of rats [45].

As the blood–retinal barrier prevents entrance of toxic molecules, plasma components and water into the retina, it restricts the passage of systemically applied drugs as well [28,35]. After systemic administration the concentration of a drug in the vitreous is typically only 1–2% of the concentration in the plasma [28]. Permeability coefficients across isolated porcine RPE tissue sheets varied from 10^{-7} cm/s for poorly permeable molecules, such as the hydrophilic beta-blocker atenolol, to 10^{-5} cm/s for highly permeable substances, such as memantine and nicotine [46]. The permeability coefficient of sodium fluorescein (1.81×10^{-7} cm/s) determined with this model was found to be in the in vivo range [46].

Like the RPE, also retinal vessel walls are poorly permeable to proteins (e.g. horse radish peroxidase) and small hydrophilic compounds (e.g. sodium fluorescein), whereas lipophilic substances can permeate retinal capillary endothelial cells more easily [22].

Many substances are eliminated from the vitreous body either by active transport or passive diffusion across the BRB. This is highly relevant in intravitreal drug administration, since high lipophilicity of the drug or the presence of an active transport mechanism lead to a rapid transport from the vitreous across the retina into the systemic blood circulation. A longer half-life in the vitreous can be observed when the passage of the drug through the BRB is not possible, and the drug has to diffuse into the anterior chamber first to be removed either by aqueous blood-flow or after diffusion across the iris surface (Fig. 1) [15]. For example, after intravitreal injection the rate of loss of gentamicin, which is removed from the vitreous via the anterior chamber, is about 0.035 h^{-1} in the rabbit, whereas for penicillin, which leaves the eye by crossing the retina, the rate of loss is 0.18 h^{-1}, so that the half-life of penicillin in the vitreous is 5 times shorter than the half-life of gentamicin [15].

3. Cell models of the anterior eye barriers

3.1. Cornea

The cornea (Figs. 1 and 2) is the major absorption route for topically applied drugs, even though it forms a tight barrier. Cell culture models of the cornea are useful tools to study transcorneal permeation of drugs in order to increase their ocular bioavailability, however, the majority of cornea models have been developed for in vitro ocular toxicity studies as an alternative to animal experimentation.

The corneal epithelium represents the rate-limiting barrier for transcorneal permeation and the uppermost epithelial cell layers cause over 60% of the total corneal resistance, for which values in the range of 3.3 ± 1.5 kΩ cm^2 [47] to 7.5 ± 0.2 kΩ cm^2 [48] are reported in the literature. Consequently, many cell culture models of the corneal barrier comprise only corneal epithelial cells [4,7,25,49–58] (Table 1). The barrier function of a cell culture model is usually characterised by electrophysiological parameters, such as the transepithelial electrical resistance (TER). The evaluation of the paracellular permeation characteristics of hydrophilic molecules is an important test for the barrier functions as well. In contrast, the permeability of lipophilic model compounds, which permeate by the transcellular way faster and to a greater extent across the cornea than hydrophilic drugs, is considerably less influenced by the tightness of the established barrier.

Numerous corneal epithelial cell cultures based on the isolation and culture of primary cells are described in the literature [49–52,59–62]. Immortalized cell lines of human corneal epithelium are available for corneal cell culture models as well [4–7]. In addition, a tissue model of the corneal epithelium for in vitro toxicity testing, which comprises immortalized human corneal epithelial cells (LSU Eye Centre, New Orleans, USA), is commercially available via SkinEthic Laboratories (Nice, France) [63].

Cell culture models of the whole cornea, consisting of epithelial, stromal and endothelial cells, have been developed to reflect the in vivo properties of the cornea more accurately [64–70]. These organotypic cornea constructs can be used for toxicity testing, and transcorneal permeation studies, and might even have the potential to be used as corneal transplants one day.

3.1.1. Corneal epithelium

3.1.1.1. Culture conditions for in vitro differentiation. The main challenge in cultivating corneal epithelium in vitro is the establishment of appropriate growth conditions for the cells, which lead to the formation of a multilayered epithelium, with the top cell layer flattened and the presence of tight junctions between these cells. In general, the closer the culture conditions are to the natural environment of the cells, the more closely the cultured epithelium will mimic the in vivo tissue.

The use of permeable support systems has proven to be a valuable tool in epithelial cell culture, as it allows cells to grow in a polarized state. These permeable membrane filters permit cells to feed basolaterally and thereby carry out metabolic activities under more natural conditions. Cellular functions such as transport, adsorption, and secretion can be studied easily since cells grown on permeable supports provide independent access to the apical and basolateral

Table 1
Cell culture models of the corneal epithelium

Species	Cell culture conditions	TER (Ω cm^2)	Characterisation	Applications	References
Primary cell culture					
Rabbit	Collagen-coated membrane; medium contains cholera toxin and 5% serum; air-lifted; culture time 8 days	~100–150	Morphology; bioelectric parameters; permeability	Permeability and active transport studies	[49–51]
Rabbit	Fibronectin/collagen/laminin-coated membrane; serum-free medium; air-lifted; culture time 7–8 days	~5000	Morphology; bioelectric parameters; keratin expression; permeability	Permeability studies	[52]
Immortalized cells					
Human (HCE-T)	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	[4,53,54]
Human (HCE)	Collagen-coated/fibroblasts membrane; medium contains cholera toxin and 15% serum; air-lifted; culture time 3–4 weeks	~400–800	Morphology; bioelectric parameters; permeability; esterase activity; paracellular pore size and density	Toxicity and permeability studies; prediction of ocular pharmacokinetics by combining in vitro permeation data with computer simulation program	[25,55–58]
Human (tet HPV16-E6/E7 transduced HCE)	Collagen-coated membrane; EpiLife medium; air-lifted; culture time 4–12 days	~400–500	Morphology; bioelectric parameters; cytogenetics; permeability	Toxicity and permeability studies	[7]

The transepithelial electrical resistance (TER) is used as parameter to characterise the tightness of the in vitro epithelium. For comparison, TER values of isolated rabbit cornea (epithelium–stroma–endothelium) were determined to be 3200–7500 Ω cm^2 [47,48].

domain. The filter material, its pore size and the matrix components on the filter are important factors in developing the cell culture model. Usually, the corneal epithelium cells are cultured on plastic supports (polycarbonate or polyester filters) with or without collagen (type I), laminin or fibronectin coating [49,52,55,71]. Collagen coating helps the cells to attach to the support and stimulates their proliferation and differentiation likewise [49,55,71]. The cell culture filters can be coated with a mixture of collagen and fibroblasts to provide a substrate resembling corneal stroma [55]. For instance, Reichl et al. used a mixture of a type I collagen and corneal stromal fibroblasts in porcine and human cornea models [69,70]. Moreover, 3T3 fibroblasts from mice as feeder layer were reported to stimulate the differentiation of the corneal epithelial cells [71,72]. Human amniotic membrane is often used when corneal epithelial cells are cultured for ocular surface reconstruction [73,74]. Its basement membrane contains collagens and several adhesive glycoproteins, which are found in corneal and conjunctival epithelial basement membranes as well [75,76].

As corneal epithelial cells are located at an air interface in vivo, the exposure to an air–liquid interface was found to be critical for the differentiation of corneal epithelial cells in culture [49,52,55,64]. Flat apical cells and proper barrier function are not formed without air-lifting [55]. A schematic presentation of a corneal epithelial cell culture model is shown in Fig. 6.

Cultivation mediums in cell culture models usually contain fetal bovine serum (FBS) in a range of 2–20% and different growth supplements such as epidermal growth factor (EGF), insulin, hydrocortisone and cholera toxin. High concentrations of FBS may in some cases disturb cell proliferation and differentiation [52,77]. Chang et al. also found out that keeping the concentration of EGF at 1 ng/ml is equally important [52]. Some groups have used serum-free medium, and they have noticed improved differentiation of corneal epithelium [4,52,53,72]. On the other hand, lower concentrations of serum were shown to decrease the surviving rate of cells [55].

3.1.1.2. Primary cell culture. Most primary cell culture models of the corneal barrier comprise corneal epithelial cells isolated from rabbits [49–52,59,78].

Kawazu et al. developed a primary rabbit corneal epithelial cell model, which was used for permeability studies and studies of active transport [49–51] (Table 1). The morphology of this model resembled an intact cornea with multilayered structure and desmosomes. After 8 days transepithelial electrical resistance (TER) reached a peak

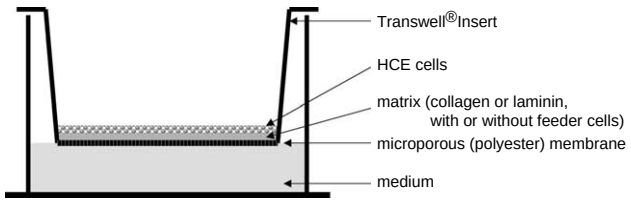

Fig. 6. Schematic presentation of the cell culture model of human corneal epithelium: HCE cells are grown on collagen-coated permeable supports after airlift.

value of 144 Ω cm^2, which, however, is far below the TER values reported for isolated rabbit cornea [47,48]. Consequently, the permeability to ^{14}C-mannitol was about 100 times higher in this model than in excised rabbit cornea [21]. According to the low electrical resistance and high permeability values for hydrophilic and lipophilic model drugs this model does not constitute a tight barrier comparable to the in vivo cornea.

Chang et al. developed a corneal epithelial membrane model with more pronounced barrier properties using primary rabbit corneal cells [52] (Table 1). After about one week the cultured cells had formed a multilayered structure with tight junctions and a peak TER value of 5000 Ω cm^2. The permeability of the hydrophilic model drug fluorescein (P_{app} $(0.32 \pm 0.01) \times 10^{-6}$ cm/s) was in the same range as the permeability in isolated rabbit cornea (P_{app} $(0.43 \pm 0.09) \times 10^{-6}$ cm/s) [79].

In numerous studies primary human corneal epithelial cells were utilized for preparing tissue sheets intended for the reconstruction of the ocular surface in the treatment of severe ocular surface disorders [74,80,81]. For instance, Ban et al. cultured human corneal limbal cells on denuded human amniotic membrane, additionally using a 3T3 fibroblast feeder layer [74]. After 28 days in culture and airlift the cells had formed a stratified epithelium with tight junctions, which was impermeable to horse radish peroxidase.

To date, primary human corneal epithelial cells are not routinely used as in vitro model for the corneal barrier, as immortalized human cell lines are generally considered to be more suitable for this purpose [4,7,53–55]. However, the further development of these immortalized cell culture models would greatly benefit from the possibility to compare their characteristics, such as presence of active transporters, with a well-established primary cell culture model of the human corneal epithelium.

3.1.1.3. Immortalized cell lines. Numerous immortalized corneal epithelial cell lines have been established from rabbit [82,83], rat [84], hamster [85] and human cells [4–7], but not all of them have been characterised with regard to their barrier function.

The immortalization of corneal epithelial cells from rabbits (RCE-cell line) and rats using a recombinant SV40 adenovirus has been published by Araki et al. [82,84]. Okamoto et al. developed immortalized rabbit corneal epithelial cells by transfecting primary cells with SV40 T antigen gene using a calcium phosphate precipitation technique [83]. Both immortalized rabbit corneal epithelial cell lines display, for example, desmosome formation, microvilli development, and the expression of cornea specific 64 kD cytokeratins. A widely used rabbit corneal cell line, SIRC (Statens Seruminstitut rabbit corneal cells), was shown to exhibit a fibroblast phenotype, which decreases its value as model for corneal epithelium [86].

Kahn et al. transfected human corneal epithelial cells with the SV40 T antigen plasmid pRSV-T (HCE-T cells) [4]. Cultured HCE-T cell displayed morphologic and phenotypic characteristics comparable to those of primary corneal epithelial cell cultures (Table 1). HCE-T cultures retained functional viability over a time period of 3 weeks. This cell line was used for establishing in vitro models of the corneal epithelium for ocular toxicity studies, but the applications of this cell line are limited because of virus shedding in the medium [53,54].

Later, Araki-Sasaki et al. established an SV40-immortalized human corneal epithelial cell line (HCE) [5]. An SV40-adenovirus vector recombinant was used, which lacked the origin of SV40 viral replication and did not produce any viral particles. This immortalized cell line continued to grow for more than 400 generations, exhibiting a cobblestone-like appearance similar to normal corneal epithelial cells in culture. When HCE-cells were grown on collagen-coated filters under airlift for 3–4 weeks, TER values in the range of 400–800 Ω cm^2 were reached. The cultured corneal epithelium consisted of five to eight cell layers. The most apical cell layer was flat, with tight junctions, microvilli and desmosomes [55].

To date, this is the most thoroughly characterised model of human corneal epithelium (Table 1). The HCE model has been used for determining the passive permeation of model compounds: neutral polyethylene glycol oligomers (PEGs), positively charged PEGs, beta-blockers and fluorescent markers [25,55,57]. The permeability of beta-blockers in the HCE model increased with increasing lipophilicity (log P) according to a sigmoidal relationship. The apparent permeability coefficient P_{app} of the hydrophilic model drug atenolol ($(1.24 \pm 0.40) \times 10^{-6}$ cm/s) was 12 times smaller than the P_{app} of lipophilic betaxolol ($(15.05 \pm 1.68) \times 10^{-6}$ cm/s). The HCE model seems to be equally permeable to hydrophilic drugs in comparison to isolated rabbit cornea (P_{app} atenolol 1.1×10^{-6} cm/s) [27], but only half as permeable for lipophilic drugs. This decreased permeability might be due to the fact that for very lipophilic drugs the corneal stroma becomes the rate-limiting barrier, and the permeability properties of corneal stroma and the collagen-coated filter of the HCE model differ from each other. The porosity, paracellular pore radius and number of pores of the HCE model were compared with the excised rabbit cornea [57,87]. In both cases the results were found to be similar. The pore radius ranged from 0.7 to 1.6 nm and the porosity in terms of paracellular pores was $(1.3–2.8) \times 10^{-7}$ [57,87]. The HCE model was also shown to display significant esterase activity [57]. It is known that the cornea is metabolically active and expresses esterases, peptidases and proteases, which may limit drug bioavailability [88,89]. On the other hand, esterase activity is relevant for the use of lipophilic ester prodrugs, where the biologically active compound is released in the corneal epithelium via hydrolysation by esterases [90].

In summary, the data indicate that the barrier function of the HCE model closely resembles that of the excised rabbit cornea in terms of passive permeability. In a subsequent study this model was used for determining corneal absorption and desorption rates of several beta-blockers [58]. These rate constants and other previously published pharmacokinetic parameters were applied to a computer stimulation model in order to predict the ocular pharmacokinetics of timolol after topical administration. The model, which represents the first attempt to combine cell culture methods and pharmacokinetic modelling, gave a reasonable estimate of the ocular pharmacokinetics of timolol. Possible applications of this model are formulation studies or the screening of new drug candidates.

In other studies HCE cells were also used for cytotoxicity testing [91–93] and for studies of corneal epithelial wound healing [94,95].

Offord et al. immortalized primary human corneal epithelial cells with a recombinant SV40 T antigen retroviral vector defective for viral replication [6] (Table 1). This immortalized cell line (CEPI-17-CL4) showed a typical cobblestone morphology, and grew over more than 200 passages. The expression of cytokines, growth factors, and metabolic enzymes was similar in these cells and human corneal epithelial cell samples [6,96]. This cell line was also used in cornea construct studies [70].

Recently, Mohan et al. developed tetracycline-responsive human papilloma virus (HPV) 16-E6/E7 transduced human corneal epithelial cell clones [7] (Table 1). The cells show tightly regulated inducible proliferation, relatively normal corneal epithelium differentiation, and typical morphology. The cells were also shown to retain several functions of untransformed corneal epithelial cells such as susceptibility to growth factor, alteration in growth and differentiation by ionic calcium, stratification, and normal cellular organization.

None of the corneal epithelial models based on CEPI-17-CL4 cells or tet HPV16-E6/E7 transduced HCE clones was examined for the barrier functions. The developers of these models focused more on the applicability for in vitro toxicity testing and in vitro studies of cell physiology. At the moment none of the corneal epithelial models, not even the HCE model, has been extensively characterised with regard to expression of metabolic enzymes, active transporters or efflux pumps. For the further development of these models such characterisation would be very important. Surprisingly, the profiles of metabolic enzymes and transporters have not been investigated yet in primary corneal epithelial cells or the in vivo cornea either.

3.1.2. Whole cornea models

Models of the entire cornea have been developed with the main purpose of in vitro ocular toxicity and permeability testing. Better correlation to the in vivo situation is expected in comparison to models consisting only of corneal epithelial cells. Organotypic cornea equivalents with epithelium, stroma and endothelium are constructed step by step on cell culture inserts. The epithelial cells are usually cultivated on stromal cells embedded in a collagen matrix with an underlying layer of endothelial cells.

The first in vitro reconstruction of the whole cornea was described by Minami et al. [64] using isolated bovine endothelial, stromal and epithelial cells in three-dimensional collagen gel matrix culture. The corneal epithelial cells expressed cornea specific keratin, and the epithelium consisted of 5–6 layers of superficial cells with microvilli, wing cells and basal cells.

Griffith et al. were first to use immortalized human corneal cell lines for a human corneal equivalent [65]. The cornea model was shown to be similar to human cornea in its morphology, histology, biological marker expression, transparency, ion and fluid transport, and gene expression. The response to different grades of injury in the engineered corneal equivalents was comparable to human and rabbit corneas, so that ocular irritancy testing is considered one possible application of the corneal equivalent. Later, the same group improved this model by developing a tissue engineered cornea complete with functional innervation [97].

Other in vitro models of the cornea were mainly characterised for applications in toxicity testing. These models are based on primary corneal cells from fetal pigs [66] and humans [67]. Although these corneal models may be useful in studying the corneal barrier, the barrier formation in these models has not been examined.

Tegtmeyer et al. developed a three-dimensional cornea construct using primary bovine corneal cells [68], which was used to study ocular permeation of pilocarpine hydrochloride from different formulations. The same rank order of permeabilities of pilocarpine in different formulations was established using the in vitro cornea model in comparison to excised bovine cornea. Similar results were obtained by Reichl and Müller-Goymann when developing an in vitro cornea utilizing primary porcine corneal cells, and evaluating its permeability with befunolol as model drug [69]. A complete human organotypic cornea equivalent was developed by the same group as well, using immortalized epithelial (CEP-17-CL4) and endothelial (HENC) cells, and primary human stromal cells [70]. The human cornea construct exhibited typical corneal structures such as a monolayer of endothelial cells and a multilayered epithelium with flat superficial cells. The permeabilities of three drugs (pilocarpine hydrochloride, hydrocortisone and befunolol hydrochloride) were determined and it was found that they were only about 1.6–1.8-fold higher than in excised porcine cornea. However, as neither TER measurements nor permeation studies with hydrophilic model drugs were performed, it remains unclear whether the corneal epithelium in these constructs forms a tight barrier.

All the above-mentioned cornea models are considered to be promising tools for in vitro toxicity testing. In addition, in vitro corneas based on human cells are the first

step towards the development of tissue engineered corneal transplants. For the use as in vitro models for permeability evaluation, however, more detailed investigations of the barrier functions of these models are needed.

3.2. Conjunctiva

The conjunctiva (Fig. 1) plays an important role in both the ocular and systemic absorption of topically applied drugs [15,16]. To reach the anterior eye via the non-corneal route the drug has to pass the bulbar conjunctiva, which is permeable to drugs of different size and polarity [26,98]. In vitro models of the conjunctival epithelium with barrier properties are useful tools in evaluating strategies to modulate either non-corneal or systemic drug absorption.

So far, mostly primary cell culture systems of rabbit conjunctival epithelial cells have been used for modelling the barrier function of the conjunctiva. These primary cell culture models have been constantly improved from the first liquid covered models [99–104] to the newer air–liquid interface cell culture systems [105–108]. In numerous publications these models were used for drug transport studies [100,101,103,105], for the evaluation of drug delivery systems [104], and for toxicity studies [108]. A brief summary of these in vitro models is shown in Table 2. Recently, two immortalized human conjunctival cell lines have been established and characterised as well [8,9].

3.2.1. Conjunctival epithelium models

The first functional in vitro model of the conjunctiva with replicate barrier properties was developed by Saha et al. [99]. Primary cell cultures of pigmented rabbit conjunctival cells were grown on permeable supports. After 8–10 days the resulting multilayer epithelial cultures exhibited transepithelial electrical resistance (TER), potential difference (PD), and equivalent short circuit current (I_{eq}) values closely similar to those of excised rabbit conjunctiva, and maintained these barrier properties for 4 days. The permeability characteristics of this model were evaluated in a subsequent study by the same group with low molecular weight drugs [100]. ^{3}H-Mannitol and six beta-blockers of different lipophilicity were used as model compounds. The apparent permeability coefficients of lipophilic beta-blockers (metoprolol, timolol, propranolol and betaxolol) were in the same order of magnitude as those determined via permeation studies with freshly excised rabbit conjunctival tissue. Surprisingly, for the hydrophilic drugs (atenolol and sotalol) the cultured conjunctival epithelium was 100 times less permeable than the excised tissue. The reason for this difference is not known.

The same group studied active transport mechanisms in conjunctival epithelial cells. In particular, the role of P-glycoprotein, which was detected on the apical surface of cultured cells and of the superficial cell layers in the excised conjunctiva, in the transport of cyclosporin A and other lipophilic drugs such as dexamethasone and propranolol [101,103], and the transconjunctival transport of the dipeptide L-carnosine [102] were investigated.

This conjunctival cell model was used to compare the permeability characteristics of pilocarpine hydrochloride in different formulations across ocular tissues (isolated pig cornea and sclera, rabbit conjunctiva) and cell cultures (rabbit conjunctival and corneal epithelial cells). In general, a good correlation between the cell culture models and the isolated tissues was observed, when measuring the influence of different formulation parameters (pH, tonicity, addition of benzalkonium chloride and EDTA) on the permeability of pilocarpine [104].

In order to establish a more accurate model of the conjunctiva the air interface culture (AIC) technique, which is also used in corneal cell culture [49,52,55,64], was applied to primary conjunctival cultures as well.

Yang et al. modified the primary conjunctival LCC model of Saha et al. [99] by transferring the cells to an air interface on day 4 after seeding on permeable supports [105]. Characterisation of the bioelectric parameters and transport properties of the cell model suggested a closer correlation with the intact tissue compared to liquid covered conjunctival cell culture. The transepithelial electrical resistance of rabbit conjunctival AIC was 1.1 ± 0.1 kΩ cm^2 and was not statistically significant different from the TER of excised rabbit conjunctiva (1.3 ± 0.1 kΩ cm^2) reported in

Table 2
Primary cell culture models of the conjunctival epithelium

Species	Cell culture conditions	TER (Ω cm^2)	Characterisation	Applications	References
Rabbit	Submerged culture; collagen-coated membrane; serum free medium; culture time 8–10 days	~1900	Morphology; presence of mucin-secreting goblet cells; bioelectric parameters; permeability; localization of P-gp	Permeability and active transport studies	[99–104]
Rabbit	Air interface culture; collagen-coated membrane; serum free medium; culture time 6–9 days	~1100	Morphology; bioelectric parameters; presence of mucin-secreting goblet cells; permeability	Metabolism and transport studies	[105–107]
Cow	Air interface culture; collagen-coated membrane; serum medium contains 10% serum; culture time 9–11 days	~5600	Morphology; bioelectric parameters; presence of mucin-secreting goblet cells; permeability, keratin expression	Toxicity studies	[108]

The transepithelial electrical resistance (TER) is used as parameter to characterise the tightness of the in vitro epithelium. For comparison, the TER value of excised rabbit conjunctiva was determined to be 1300 Ω cm^2 [109].

literature [109]. The P_{app} of beta-blockers varied with lipophilicity in a sigmoidal fashion, and for lipophilic beta-blockers the P_{app} was in the same range as in excised conjunctival tissue. For instance, the P_{app} of propranolol was $(0.98 \pm 0.02) \times 10^{-5}$ cm/s in the air-interface conjunctival model and $(0.79 \pm 0.08) \times 10^{-5}$ cm/s in the excised tissue. However, hydrophilic beta-blockers exhibited again significantly lower permeability in the cell culture model. The presence of active cation transporters in the normal rabbit conjunctiva is given as one possible explanation for the higher permeability of hydrophilic beta-blockers in the excised tissue. The permeability of neutral hydrophilic compounds (mannitol, FITC-dextrans) was very similar to the excised tissue (e.g. P_{app} of mannitol: $(2.18 \pm 0.15) \times 10^{-7}$ and $(2.77 \pm 0.43) \times 10^{-7}$ cm/s in the cell culture model and in excised tissue, respectively) and decreased with molecular size. When compared to the excised tissue a 2-fold higher P_{app} was measured for high molecular weight hydrophilic drugs (FD20 and FD70). This observation might be attributed to the slightly larger pores in the cell culture model (8 nm) in comparison to the excised tissue (5.5 nm) [105]. This more physiologic model was used to investigate metabolism and transport of glutathione in conjunctival epithelium [106,107].

Recently, Civiale et al. developed a cell culture model of the conjunctival tissue using bovine conjunctival cells [108]. The model was designed for toxicity studies rather than for studying conjunctival permeability. Primary conjunctival epithelial cells were isolated from bovine eyes obtained from a local slaughterhouse and grown on collagen-coated permeable supports. The model was characterised by morphological examination, measurement of bioelectric parameters, paracellular transport studies, and cytotoxicity tests. Cultures that were transferred to air interface possessed a tissue like structure and unexpectedly high peak TER values of 5.6 kΩ cm^2, which are more than 4 times higher than the mean TER value measured for excised rabbit conjunctiva (1.3 kΩ cm^2) [109]. The permeability properties of this model were not compared with those of excised bovine conjunctival tissue, so it remains unclear whether the model reflects the in vivo barrier properties of conjunctival epithelium.

All the cell culture models described in this section are models of rabbit or bovine conjunctiva. For the further development of these models their passive permeability properties need to be characterised in more detail and compared to the intact tissue. Moreover, comparison of the obtained results to the in vivo situation in humans is generally missing. In addition, very little is known about the role of transporters in the human conjunctiva in vivo. The use of human conjunctival cells will be the next step in establishing a functional in vitro model of the conjunctival barrier. Methods for primary human conjunctival cell culture have already been described, and they are currently used for conjunctival tissue transplantation in the treatment of severe ocular surface disorders [110,111]. Furthermore, two immortalized human conjunctival cell lines have become available in the meantime as well [8,9], but so far they have not been used for developing a model of human conjunctival epithelium.

4. Models of blood–ocular barriers

4.1. Blood–aqueous barrier

In contrast to numerous established cell culture models of the blood–retinal barrier, functional cell culture models of the blood–aqueous barrier are not described in the literature.

One primary cell culture model of rabbit ciliary body non-pigmented epithelial cells was developed by Cilluffo et al. [112], but the TER of the cell layers was only 20–30 Ω cm^2 due to the presence of spaces between the cells. In recent studies, Cilluffo et al. used isolated rabbit ciliary body non-pigmented epithelial cells for studying changes of intracellular free Ca^{2+} [113,114]. Monolayers of human non-pigmented ciliary epithelium cultured by Noske et al. exhibited only incomplete belts of tight junctions, which resulted in a low transepithelial resistance of 19.7 ± 2.1 Ω cm^2 [115]. To our knowledge, a cell culture model for the evaluation of the barrier functions of iris capillary walls has not been described in the literature.

4.2. Blood–retinal barrier

Numerous cell culture models have been established as models of the inner and outer blood–retinal barrier. They are useful tools in studying the cell biology and, in particular, the transport functions of the RPE and the retinal capillary endothelial cells. For instance, the RPE mediates the transport of nutrients, metabolites, ions and fluid between the neural retina and the choroid, which is essential for the viability of the retina. Special emphasis is also placed on the investigation of the mechanisms of blood–retinal barrier breakdown, which occurs in diseases such as diabetic retinopathy, retinitis pigmentosa, uveitis, and age-related macular degeneration [116]. Any disruption of either the inner or the outer blood–retinal barrier results in the development of retinal edema and can lead to a loss of vision [35].

In addition, these cell culture models can be used for the development of drug delivery systems targeted to the posterior segment of the eye.

4.2.1. Retinal pigment epithelium

Due to the many specialized features of RPE, the establishment of a cell culture system that is an appropriate in vitro model for the barrier function of the RPE remains a scientific challenge. Cell culture conditions can alter the morphological and functional appearance of the cultured cells [117]. For example, unless the appropriate cell culture conditions are used, the cells lose their polarized features.

In addition, RPE cells proliferate only in pathologic conditions in vivo. For in vitro cell culture the induction of proliferation in RPE cells depends on various factors, such as the species, donor age, isolation process time, and the culture conditions [116].

4.2.1.1. Primary cell culture. Primary cell culture models of the RPE utilizing frog [118,119], rat [120,121], chick [122–125], bovine [126] and human RPE cells [127–129] have been described in the literature. A brief summary of these in vitro models of the RPE barrier is shown in Table 3.

In comparison to human donor eyes, continuous supply of animal tissue for cell isolation can be more easily arranged. Nevertheless, most researchers focus on the use of human RPE cells to avoid species related applicability problems. For instance, primary cell culture models of human RPE were used for studies of uptake and transport [130–133], and protein expression [133,134], for the evaluation of delivery systems for genes and antisense oligonucleotides [135–137], and for cytotoxicity studies [138–140].

4.2.1.2. Immortalized cell lines. Different continuous cell lines (rat and human) have been established of retinal pigment epithelial cells [10,11,141–143]. The cell lines differ from each other in various properties and an adequate in vitro model of the barrier function of the RPE has yet to be established.

The rat RPE-J cell line was produced by infection of rat RPE cells with a temperature sensitive SV40 virus and subsequent isolation of epithelial clones [142]. The cells were shown to exhibit a highly differentiated phenotype in culture, but the polarity of Na^+, K^+-ATPase and the neural cell adhesion molecule N-CAM differs from the in vivo localization.

Davis et al. cloned a spontaneously arising cell line (D407) from a primary culture of human retinal pigment epithelium [10]. The cells were shown to possess most of the metabolic and morphologic characteristics of RPE cells in vivo, such as an epithelial cobblestone morphology, expression of typical keratins, and synthesis of retina specific CRALBP protein. However, the loss of some enzymatic activities and of cytoskeletal polarization was observed. Consequently, the cell line is mainly used for cytotoxicity studies, as model for retinal metabolism, and for retinal gene delivery studies [144–148], and not as model for barrier and transport function.

The second human RPE cell line (ARPE-19) was established and characterised by Dunn et al. [11]. The cell line, which arose spontaneously, was purified until a highly

Table 3
Cell culture models of the retinal pigment epithelium (RPE)

Species	Cell culture conditions	TER (Ω cm^2)	Characterisation	Application	References
Primary cell culture					
Xenopus laevis (African clawed frog)	Matrigel coated filters; serum free defined medium; culture time 2–4 weeks	~400	Morphology, electrophysiological characterisation, distribution of Na^+, K^+-ATPase	Membrane polarity of Na^+, K^+ pump, retina–epithelium interactions	[118,119]
Rat	Laminin-coated filters; serum-free medium; culture time 5–7 days	~200	Morphology, bioelectric parameters; permeability	Influence of serum on tight junction formation	[120]
PVG rat	Uncoated filters; medium contains 2% serum; culture time 3 days	~70	Permeability, bioelectric parameters; tight junction morphology	Effect of cytokines and NO on permeability	[121]
Chick embryo	Laminin-coated filters; serum free medium; retinal-conditioned medium; culture time 9 days	40–140[a]	Morphology, bioelectric parameters; permeability, distribution of ZO-1, actin	Development and regulation of tight junctions; influence of medium composition	[122–125]
Cow	Uncoated filters; co-culture with endothelial cells; culture time 14 days	~200	Permeability, bioelectric parameters; expression of VEGF	Effect of endothelial cells on barrier function of RPE	[126]
Human	Matrigel coated filters; medium contains 1% serum; assays performed after 19 days in culture	~30	Morphology, bioelectric parameters; permeability, secretion of cytokines after stimulation	Polarized secretion of cytokines; comparison between ARPE-19 cell line and human RPE cells	[127]
Human fetal	Millicel PCF filters, CEM replacement medium; culture time 1 month	~530	Permeability, bioelectric parameters; expression of tight junction proteins	Influence of Na^+, K^+-ATPase on tight junction function	[128,129]
Immortalized cells					
Human ARPE-19 cells	Laminin-coated filters; medium contains 1% serum, culture time 4 weeks	~100	Morphology, bioelectric parameters; permeability, expression of marker proteins	Characterisation of monolayers formed by ARPE-19 cell line	[11]

The transepithelial electrical resistance (TER) is used as parameter to characterise the tightness of the in vitro epithelium. For comparison, a mean TER value of 148 Ω cm^2 was reported for human RPE explants [43].

[a] Depending on culture conditions.

epithelial culture of RPE cells with a strong growth potential was obtained. The cells were characterised with regard to their morphology, the expression of retina specific markers (CRALP and RPE65), and their barrier properties (Table 3). The authors found that ARPE-19 cells retained characteristic morphological features such as defined cell borders, a 'cobblestone' appearance and pigmentation. Both RPE specific markers were detected in the cells. When plated on laminin-coated Transwell-COL filters the ARPE-19 cells exhibited morphological polarization. The barrier properties were evaluated by measuring the transepithelial electrical resistance (TER) and the permeability of ^{3}H-inulin. When the cells were grown in a specialized medium for RPE cell culture instead of DMEM-F12 plus 1% FBS medium, higher TER values were measured and the percentage of apical inulin appearing in the basolateral chamber was decreased from 10.9 to 5.9%. However, although tight junctional complexes were located at the apical pole of the cells, measured TER values were only 50–100 $\Omega\,cm^2$ [11]. In a subsequent study by Dunn et al. the applicability of the ARPE-19 cell line as model for RPE polarity studies was investigated further [149]. The authors used viral gene transfer methods to express well-characterised exogenous proteins in ARPE-19 cell monolayers. An examination by confocal microscopy and surface labelling techniques showed that ARPE-19 cells display a polarized distribution of cell surface markers. The authors conclude that this cell line provides a suitable model for polarity studies in RPE cells.

Monolayers of ARPE-19 cells have become a well-established in vitro model of the outer blood–retinal barrier. For example, this cell culture model was used for uptake and transport studies with [^{3}H]-verapamil [150] and endogenous substances [151,152]. The influence of interleukin-1β and oxidative stress on the barrier function of the RPE was evaluated with this in vitro model as well [153,154]. It could be shown that oxidative stress negatively affects RPE cell junction and barrier integrity, which may contribute to the pathogenesis of age-related macular degeneration [154]. Moreover, monolayers of ARPE-19 cells were used by researchers for a variety of other in vitro experiments, including studies of the regulation of gene expression [13,134], polarized distribution and secretion of proteins [155], delivery of genes and antisense oligonucleotides [156,157], for toxicity studies [158,159], and as models of retinal diseases [154,160].

A third immortalized cell line of human RPE (hTERT-RPE) was established by Bodnar et al. by transfecting RPE cells with vectors encoding the human telomerase catalytic subunit [143]. With this method the cellular life span, which in absence of telomerase is limited due to telomer loss, can be extended while the cells maintain the diploid status, growth characteristics and gene expression pattern of normal cells [143,161]. Rambhatla et al. investigated the cell culture conditions necessary for the differentiation of TERT-RPE cells [162]. The cells were shown to undergo growth arrest and differentiation in the absence of serum. After 4 weeks and more in serum free culture the cells had formed a monolayer of homogenous and melanin containing cells expressing RPE-associated proteins [162].

Among the disadvantages of the so far described cell culture models of human RPE are the long culture duration of up to 2 months described by some authors [126,163], during which the cells might lose their tissue specific features, and also their probably not adequate barrier functions as indicated by TER values of only 30–50 $\Omega\,cm^2$ [127,149]. In comparison, mean TER values of 206 and 148 $\Omega\,cm^2$ were reported for RPE explants of human fetal and human adult eyes, respectively [43]. The limited availability of human donor eyes is another drawback in the development of in vitro models of the blood–retinal barriers, because primary cell culture models are valuable for comparison in the characterisation of immortalized RPE cell lines. In addition, more information about the active transport mechanisms and passive permeability across the RPE in vivo is necessary to validate the cell culture models with regard to their in vitro/in vivo correlation.

4.2.2. Retinal capillary endothelium

The breakdown of the inner blood–retinal barrier occurs in several pathologic conditions of the eye, including diabetic retinopathy. In order to find new treatment strategies for these diseases researchers are focussing on identifying factors that influence permeability of the retinal capillary endothelium. The established in vitro models utilizing bovine retinal capillary endothelial cell (BRCEC) culture do not completely reflect the barrier function of the human retinal capillary endothelium in vivo, however, by the use of these models changes in permeability can be measured more easily and more precisely than with in vivo studies [164–171].

4.2.2.1. Primary cell culture. Gillies et al. grew second passage bovine retinal capillary endothelial cells on polycarbonate filters coated with gelatin, laminin, fibronectin and collagen [172]. The cells were characterised with regard to the bioelectrical properties, permeability of inulin and expression of blood–brain barrier related enzymes. After about 7 days the cell layer exhibited a mean peak resistance (TER) of $129 \pm 32\,\Omega\,cm^2$ and the P_{app} of inulin was $(1.7 \pm 0.9) \times 10^{-7}$ cm/s. Results of permeability and enzyme expression studies indicated that the cells retained their specialized barrier properties when grown as monolayer in vitro. In subsequent studies, Gillies et al. studied the effects of interferon-α2b and high glucose levels on the permeability of the BRCEC monolayer [164,165].

In an attempt to increase the barrier resistance of this cell model Tretiach et al. co-cultured Müller (glial) cells with bovine retinal capillary endothelial cells, since Müller cells in the retina are involved in the inner blood–retinal barrier function [173]. The cells were characterised by electrical

resistance studies, immunocytochemistry and transmission electron microscopy. A severe limitation of the model was that the cells commonly grew in multilayers instead of a monolayer. In addition, the TER values of Gillies et al. [172] could not be reproduced. Because of irregular cell growth many preparations did not form a functional barrier [173]. Despite the irregular growth of BRCECs in vitro, Tretiach et al. could show in a subsequent study that by using medium conditioned by mixed RPE cells and Müller cells, the inulin and albumin permeability of bovine retinal capillary endothelial cell monolayers was significantly decreased in comparison to monolayers cultured in unconditioned medium. In addition, the TER increased about 4-fold, reaching values of about 40 $\Omega\,cm^2$ [174].

Gardner et al. developed a bovine retinal epithelial cell culture system for studying the mechanisms of histamine increasing endothelial permeability [166,167]. The model was modified by Yaccino et al. to investigate the physiological and pathophysiological functions of vascular endothelial growth factor (VEGF). However, the cells did not form a tight barrier and reached a maximum TER of only 25 $\Omega\,cm^2$ about 10 days after plating. The P_{app} of albumin was $(1.95 \pm 0.29) \times 10^{-6}$ cm/s [168]. The mechanisms by which VEGF increases BREC permeability were further investigated utilizing this model by Chang et al. [169] and Lakshminarayanan et al. [170].

Antonetti et al. characterised the influence of hydrocortisone on the barrier properties of this BREC monolayer model and it was shown that hydrocortisone reduces endothelial permeability by decreasing occludin phosphorylation, and increasing occludin content and tight junction assembly [171].

4.2.2.2. Immortalized cell lines. Recently, a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB) was developed by Hosoya et al. from a transgenic rat harboring the temperature sensitive SV40 T antigen gene [175]. This cell line exhibits temperature dependent cell growth and was shown to have endothelial properties.

Shen et al. evaluated the barrier properties of the TR-iBRB cell line by growing the cells on permeable supports for drug transport studies [176]. Cells grown on fibronectin-coated filters showed morphologic characteristics similar to that of endothelial cells in primary culture. However, the cells did not form tight barriers as indicated by the low TER values (30 $\Omega\,cm^2$), which are in the range of TER values achieved with primary endothelial cell culture [168]. Permeation studies were performed with numerous model substances including beta-blockers of different lipophilicity, ^{14}[C]-3-*O*-methyl-D-glucose and rhodamine 123. Rhodamine 123, which is a P-glycoprotein substrate, was also used for cellular uptake experiments. Results of these transport studies showed that with this endothelial cell model compounds can be differentiated in correlation to their physicochemical properties, and by their transport via active transporters present in the inner blood–retinal barrier. However, the P_{app} values of the beta-blockers were generally high and permeability of the most lipophilic beta-blocker (alprenolol; $P_{app} = 1.04 \times 10^{-4}$ cm/s) was only 2 times higher than the permeability of the most hydrophilic beta-blocker (atenolol; $P_{app} = 0.51 \times 10^{-4}$ cm/s) [176].

In a recent study Hosoya et al. characterised active transport mechanisms for vitamin C across the inner BRB in vitro and in vivo [177]. Results of uptake studies with TR-iBRB monolayers indicated that vitamin C is mainly transported as dehydroascorbic acid across the BRB, and these findings were in good agreement with the results of the in vivo studies in rats.

So far, a cell culture system of retinal capillary endothelial cells that models the in vivo barrier function has not been established. In comparison, much more and better characterised in vitro models have been developed of the similar blood–brain barrier (BBB). However, the establishment of a functional in vitro BBB model has been met with difficulties as well, because the genes expressing the numerous transporters and enzymes in capillary endothelial cells are repressed when the endothelial cells are grown in culture. Therefore, in vivo BBB permeability could not be reliably predicted from the results obtained. Co-culture with astrocytes and/or pericytes was shown to increase TER and decrease paracellular transport across the BBB model to some extent [178]. More importantly, a conditionally immortalized cell line has been established, which retains in vivo transport properties and gene expression of native brain microvessel endothelial cells [179].

For the establishment of an in vitro model of the inner BRB, which can be used for predicting drug permeability, a more detailed characterisation of cell lines with regard to the expression of transporters, and eventually co-culture with glial cells will be necessary. The barrier function of existing models needs to be more systematically characterised, for example in terms of passive permeability of drugs as a function of their molecular weight and lipophilicity. The further development of models of the inner BRB will moreover require a better knowledge of the in vivo barrier function of the retinal capillary endothelium, including the determination of in vivo permeability values.

5. Conclusions

During the last years much progress has been made in the establishment of cell culture models of the ocular barriers. These in vitro models were used in different research fields, including studies of cell physiology, studies of drug uptake and transport, investigation of pathological conditions, toxicological screening of compounds as alternative to in vivo toxicity tests, and tissue engineering. They are considered to be valuable tools in the development of treatment strategies for ocular diseases, such as diabetic

retinopathy or age-related macula degeneration, which are major causes for visual impairment.

However, much work still needs to be done in order to develop in vitro models of the ocular barriers that adequately reflect the characteristics of their in vivo counterparts. Before such models can be reliably used for pharmaceutical testing and in vivo predictions a more systematical characterisation of the existing models is needed. Even more importantly, in many cases a better knowledge about the functions of these barriers in vivo is required.

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