Human corneal equivalent as cell culture model for in vitro drug permeation studies

S Reichl, J Bednarz, C C Müller-Goymann

Aims: For the study of transcorneal in vitro permeation of ophthalmic drugs, excised animal cornea or corneal epithelial cell culture are frequently used as a replacement for the human cornea. The main purposes of this study were to reconstruct a complete human organotypic cornea equivalent, consisting of all three different cell types (epithelial, stromal, and endothelial); to test the barrier function of this bioengineered human cornea using three different model drugs (pilocarpine hydrochloride (PHCl), befunolol hydrochloride (BHCl), and hydrocortisone (HC)); and to determine its usefulness as an in vitro model for prediction of ocular drug absorption into the human eye.

Methods: A multilayer tissue construct was created step by step in Transwell cell culture insert using SV-40 immortalised human endothelial and epithelial cells and native stromal cells (fibroblasts). Morphology was characterised by light microscopy using routine H&E staining. Scanning electron microscopy was used to evaluate ultrastructural features. Ocular permeation of drugs across the human cornea construct was tested using modified Franz cells and compared with data obtained from excised porcine cornea and previously described porcine cornea constructs.

Results and conclusion: The cornea construct exhibited typical corneal structures such as a monolayer of hexagonally shaped endothelial cells and a multilayered epithelium consisting of seven to nine cell layers with flat superficial cells. The formation of microplicae and microvilli was also confirmed. The human cornea construct showed similar permeation behaviour for all substances compared with excised porcine cornea. However, permeability (permeation coefficients Kp) of the human cornea equivalent (PHCl 13.4 \( \times 10^{-6} \) (SD 3.01 \( \times 10^{-6} \)); BHCl 9.88 \( \times 10^{-6} \) (SD 1.79 \( \times 10^{-6} \)); HC 5.41 \( \times 10^{-6} \) (SD 0.40 \( \times 10^{-6} \)) cm/s) was about 1.6–1.8 fold higher than excised porcine cornea. Compared with data from the porcine cornea construct the cultivated human equivalent showed a decreased permeability. The reconstructed human cornea could be appropriate to predict drug absorption into the human eye.
MATERIAL AND METHODS

Materials
Borocarpin S 2% from Dr Winzer (Olching, Germany) containing 2% pilocarpine hydrochloride (0.01% benzalkonium chloride as preservative) and Glaunconex 0.5% (AlconPharma, Freiburg, Germany) containing 0.5% befunol hydrochloride (0.007% benzalkonium chloride) are commercial aqueous eye drop solutions. HC 0.02% OK is an aqueous solution of 0.02% hydrocortisone.

Cell culture
For the isolation of primary fibroblast cultures, human corneal scleral rings were received from the cornea bank, Hannover, Germany. To obtain corneal fibroblasts, both endothelial and epithelial sheets as well as scleral tissue were dissected off. The stroma was cut into small pieces and 3 mm explants were attached to a 100 mm diameter plastic dish (Costar, Fernald, Germany). Fibroblasts showed outgrowth after 6–7 days. Dulbecco’s modified Eagle’s medium DMEM supplemented with 10% fetal calf serum FCS, 4 mM L-glutamine and 1% antibiotic/antimycotic solution (Gibco BRL Life Technologies, Karlsruhe, Germany) supplemented with 5% FCS (Biochrom) and 1% antibiotic/antimycotic solution (Gibco BRL). The immortalisation of primary human corneal epithelial cells by a recombinant simian virus (SV) 40 T-antigen retroviral vector defective for viral replication, as well as characterisation of the transfected cells, has been described earlier. Cells of this cell line (CEPI 17 CL 4) from passage 29 were used. The CEPI cells were grown in medium DMEM/F12 for additional 7 days submerged to confluence. After contraction collagen lattice and grown in medium DMEM/F12 for additional 7 days submerged to confluence. After contracted collagen lattice and grown in medium DMEM/F12 the stromal cells.

Light microscopy
Tissue was fixed in a 75 mM phosphate buffer pH 7 containing 3.5% formaldehyde, dehydrated in isopropyl alcohol, and embedded in paraffin. Cross sections of 4 μm were cut, stained in hematoxylin eosin and examined with a photomicroscope Olympus IX50 (Olympus, Hamburg, Germany).

Scanning electron microscopy
Samples were fixed with 1.5% paraformaldehyde, 3% glutaraldehyde, 1.5% acrolein in 0.2 M cacodylate buffer (pH 7.2) for 24 h at 20°C, treated with 2% glycerine, sodium glutamate, sucrose (pH 6.2) for 12 h at 20°C, then with 2% tannic acid pH 4 for 12 h at 20°C, rinsed in H2O and postfixed with 2% osmium tetroxide for 12 h at 20°C. Tissue equivalent was dehydrated in a graded series of isopropyl alcohol and sputtered with gold particles before examination in a Stereoscan 250 (Cambridge Instruments, Cambridge, UK) scanning electron microscope.

Permeation studies
Diffusion experiments for the evaluation of transcorneal drug permeability from three aqueous solutions containing model drugs (PHCl, IIC, BHCJ) were performed for 420 minutes using modified Franz diffusion cells at 37°C. To compare the barrier function, both excised porcine cornea and organotypic human cornea construct were used. The donor drug formulations used were Borocarpin S 2%, HC 0.02% OK and Glaunconex 0.5% as described above. The receiver solution contained isotonic phosphate buffered saline pH 7.4 and was stirred with a magnetic stirrer (Janke&Kunkel, Staufen, Germany) at 400 rpm during the experiment. Samples were taken from the receiver chamber at fixed time intervals (every 60 min) and quantitatively analysed by high performance liquid chromatography (HPLC).

The permeation parameters of model drugs were calculated by plotting the amounts (μg/cm²) of drug permeated through the excised cornea or cornea construct versus the time (minutes). The permeation coefficient Km was calculated as flux/drug concentration from the linear ascent of a permeation curve.

HPLC methodology
Concentrations of model drugs in the samples were determined using a Waters 515, 717 plus, 486 HPLC system (Waters, Eschborn, Germany) at ambient temperature, using columns of Gromsil 120 ODS-3 CP 5 μm, 125×4 mm (Grom, Herrenberg, Germany) in the case of pilocarpine hydrochloride and befunol hydrochloride, while a column of Hypersil ODS 3 mm (Waters) in the case of Glaunconex 0.5% and hydrocortisone. The standard HPLC methods used for pilocarpine hydrochloride, befunol hydrochloride, and hydrocortisone have already been described earlier. Data analysis and calculation were performed by Waters Millenium 32 Chromatography Manager software (Waters).

RESULTS

Corneal cells
Native human fibroblasts were obtained by standard outgrowth technique. Purity of cell population was ensured by specific isolation method and indicated by typical fibroblastoid appearance of cells. Stromal cells in culture are elongated and spindle shaped as shown in figure 1B. Cells showed a good proliferation rate and could be cultivated for 10 passages without any change of their typical morphology.
The SV-40 transformed endothelial (HENC) and epithelial (CEPI) cells are well characterised immortalised cell lines and show a high proliferation rate. HENC showed morphological similarities to endothelial cells in vivo and were able to form a tight monolayer of hexagonally shaped cells (fig 1C) which closely resemble human corneal endothelium in vivo. The morphological and functional properties of these cells after transplantation onto donor corneas have been described before. Furthermore CEPI expressed the cobblestone like pattern which is characteristic of corneal epithelial cells in culture. Figure 1A shows a monolayer of polygonally shaped epithelial cells.

**Cornea equivalent**

The in vitro cornea was assembled in Transwell culture insert to ensure both a submerged cultivation and a differentiation of multilayered epithelium by growth at the air-liquid interface. The completely grown bio-engineered human cornea construct (HCC) consisted of a stroma equivalent containing native fibroblasts, an underlying endothelium, and a stratified epithelium on top derived from HENC and CEPI 17 CL 4 respectively. Figure 2 shows different phases in the stepwise reconstruction of HCC. After seven days of cultivation, a confluent monolayer of endothelial cells grown on collagenous sheet occurred. A tight mosaic like pattern of hexagonally shaped endothelial cells was clearly visible (fig 2A). The morphology of cultured endothelial monolayer was similar to the typical structure of human corneal endothelium in vivo. Within a further 4–5 days the stroma equivalent was cultivated consisting of fibroblasts embedded in a collagen gel. Within 1–3 days of cultivation, contraction of gel depending on collagen concentration and number of cells was observed, as well as spreading out of the stromal cells in the collagen lattice (fig 2B). After seeding of epithelial cells on stroma biomatrix and cultivation for additional seven days light microscopy analysis indicated that cultured corneal epithelial cells on the biomatrix had formed a tight monolayer (fig 2C).

For expression of a multilayered epithelium, the cornea construct was cultivated at air-liquid interface and microscopic examination of hematoxylin and eosin (H&E) stained cross sections of HCC were performed after ten further days of cultivation. Figure 3B shows the superior part of human cornea construct consisting of stratified epithelium grown on stroma equivalent. A tightly packed epithelium of seven to nine cell layers was detectable. The cultivated epithelium closely resembled the corneal epithelium found in porcine cornea in vivo (fig 3A) but in the case of reconstructed epithelium it was difficult to distinguish the basal cells from the wing cells. However, the uppermost cells (superficial cell
layer) appeared flattened as is also the case in vivo. This finding is in agreement with other reconstructed human corneal epithelium containing transformed epithelial.17 27 28

Scanning electron microscopy experiments also showed similarities in morphology between reconstructed tissue and the cornea in vivo. A confluent monolayer was formed by seeding human endothelial cells onto a collagen gel. The monolayer exhibited a predominantly physiological structural organisation similar to normal human endothelium.

**Figure 4** Scanning electron microscopic images of (A) cultured endothelial cells confluent grown on a type I collagen gel (scale bar = 10 μm), and (B) epithelial surface of cornea construct (scale bar 5 μm).

**Figure 3** Histological cross sections of (A) original porcine corneal epithelium and (B) cultured human epithelial cells grown on a stroma equivalent and underlying endothelial sheet stained with hematoxylin eosin (scale bar = 25 μm).

**Figure 5** Permeated amounts of (A) pilocarpine hydrochloride (PHCl), (B) befunolol hydrochloride (BHCl), and (C) hydrocortisone (HC) versus time across human cornea construct (HCC), porcine cornea construct, and excised porcine cornea. Each point represents the mean (SD) of 5–12 determinations.

**Table 1** Drug permeability of HCC compared with porcine cornea construct and excised porcine cornea. The permeation coefficients \(K_p\) (cm/s) of pilocarpine hydrochloride (Borocarpin S 2%), befunolol hydrochloride (Glaunex 0.5%), and hydrocortisone (HC 0.02% OK), mean (SD) \((n=5-12)\).

<table>
<thead>
<tr>
<th>Donor</th>
<th>Log (P^*)</th>
<th>Excised porcine cornea</th>
<th>Human cornea construct</th>
<th>Porcine cornea construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borocarpin S 2%</td>
<td>-1.3</td>
<td>(8.57\times10^{-8}) (0.53\times10^{-6})</td>
<td>(1.34\times10^{-5}) (3.01\times10^{-8})</td>
<td>(3.56\times10^{-6}) (1.53\times10^{-8})</td>
</tr>
<tr>
<td>Glaunex 0.5%</td>
<td>-0.4</td>
<td>(5.95\times10^{-6}) (0.92\times10^{-6})</td>
<td>(9.88\times10^{-8}) (1.79\times10^{-8})</td>
<td>(2.48\times10^{-6}) (4.79\times10^{-8})</td>
</tr>
<tr>
<td>HC 0.02%</td>
<td>1.6</td>
<td>(3.07\times10^{-5}) (0.49\times10^{-6})</td>
<td>(6.41\times10^{-6}) (0.40\times10^{-6})</td>
<td>(7.11\times10^{-6}) (1.21\times10^{-6})</td>
</tr>
<tr>
<td>OK</td>
<td></td>
<td>(0.99\times10^{-5})</td>
<td>(0.40\times10^{-6})</td>
<td>(1.21\times10^{-6})</td>
</tr>
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</table>

Cells displayed the characteristic hexagonal form and junctional complexes between neighbours appeared well developed (fig 4A). Furthermore figure 4B shows a micrograph of the epithelial surface of HCC. Superficial epithelial cells also formed tight sheets and typical structures such as microvilli and microplacae were visible. But obvious distinctions to the epithelium in vivo were observed. Compared with in vivo epithelium, cultured epithelium exhibited fewer microvilli and microplacae, and the interface between adjacent cells appeared less well defined. In addition cultivated epithelial cells seemed to be less flattened than superficial cells in vivo.

Permeation studies
In order to investigate the potential of HCC as an in vitro model for transcorneal permeation studies, the permeation of three ophthalmic model drugs was evaluated and compared with that via excised porcine cornea and a previously developed organotypic porcine cornea construct. The permeation profiles of each model drug through different cornea constructs and excised cornea are presented in figure 5. The resulting permeation coefficients $K_p$ were expressed as mean (standard deviation) and are shown in table 1. For all drugs the same permeation ranking with the different corneal tissues was observed. The highest permeability was detectable with the porcine cornea construct. Excised porcine cornea appeared least permeable for the drugs, although differences in permeability towards HCC were fairly small. Furthermore HCC showed distinctly increased barrier properties in comparison with the porcine cornea construct. It should be pointed out that permeability of HCC in comparison with excised porcine cornea was merely increased by factor 1.6 to 1.8 for all three drugs.

DISCUSSION
Extensive research is being done at present in the field of three dimensional tissue engineering, as well as in the development of cell culture models of biological barriers as in vitro test systems for drug absorption and delivery. Corneal epithelial cultures or co-cultures of epithelial cells and fibroblasts have recently been examined in in vitro permeation studies. Furthermore reconstruction of animal corneal tissue has been described and described for the first time a complete functional human corneal epithelium equivalent constructed from cell lines including all three corneal cell types. To our best knowledge, the use of a complete human corneal equivalent with epithelial, stromal, and endothelial cells in drug permeability studies to assess its usefulness as an in vitro model for permeation studies. Furthermore HCC should be promising in in vitro toxicological studies.

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