


Corneal Models for the Toxicity Testing of Drugs and Drug Releasing Materials

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Summary

The Draize rabbit eye irritation test developed in the 1940's is even today the only eye toxicity test officially accepted in the OECD countries for regulatory purposes in the classification of slightly and moderately irritating chemicals. It is based on the subjective scoring of three tissues of the eye: the cornea, the conjunctiva and the iris. The Draize test has been widely criticized for both scientific and ethical reasons, and alternatives have been investigated for several decades. In fact, it has been estimated that more effort has been focused on finding alternatives to the Draize eye test than on all the other acute in vivo toxicity tests combined. Organotypic test models, i.e. isolated rabbit and bovine whole eyes and corneas and various kinds of cell culture techniques have been developed to replace the Draize eye test. Extensive research in the field of tissue engineering has concentrated on the development of an in vitro model for the cornea. These threedimensional corneal models were constructed by tissue engineering methods from the three corneal cell types: epithelial cells, keratocytes, and endothelial cells, and they provide a promising tool for the testing of the corneal toxicity of drugs and drug releasing materials.

KEYWORDS: corneal model, Draize eye test, eye irritation, ocular toxicity, alternatives

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INTRODUCTION

Tissue and organ transplantation offer great opportunities in modern medicine. However, one of the main problems is the availability of transplantable tissues and organs. To overcome this problem, tissue engineering is being investigated to reconstruct new tissues and, ultimately, whole new organs. Besides transplantation, tissue engineering techniques can be used in other biomedical applications, *e.g.* drug permeation studies, and in toxicology as an alternative for animal experimentation. The Draize rabbit test (1), developed in the 1940's, is the only eye toxicity test officially accepted in the Organization for Economic Co-operation and Development (OECD) Guidelines (2) for regulatory purposes in the classification of slightly and moderately irritating chemicals. The 15th Addendum to the OECD Guidelines for the Testing of Chemicals (3) involves *in vitro* tests for phototoxicity and skin corrosion testing (transcutaneous electrical resistance test and human skin model test) which can be included in sequential testing strategies in eye irritation evaluation. The *in vivo* rabbit test only needs to be performed as a last step, when safety assessments in all the other tiers by relevant *in vitro* tests have produced negative results. A variety of different scoring systems assessing the extent of injury to the corneal, the iridial and the conjunctival compartments of the eye are currently applied in different regulations ranging from the single tissue scores to the average weighed sum scores of all the tissues. The Draize eye test is the most widely criticized single toxicity test, and it has been estimated that more effort has been focused on finding alternatives to the Draize eye test than on all the other acute *in vivo* toxicity tests combined (4). The most recent validation studies have shown that no present single test, combination of tests, or testing strategy of *in vitro* alternative methods is capable of replacing the Draize eye test completely (5). Organotypic test models, those using isolated rabbit and bovine whole eyes and corneas, have been shown to be the most potential alternatives. Extensive research in the field of tissue engineering has been focused on the development of an *in vitro* model of the cornea. Reconstructed three-dimensional corneal equivalents are a promising tool, especially for testing the toxicity of drugs and drug releasing materials. In addition, these models of the cornea are useful tools to study the transcorneal permeability and ocular bioavailability. In the future, engineered tissue corneas may also have potential to be used as transplants.

THE CORNEA

The tear-fluid covered cornea forms the outer part of the eye globe that is exposed to the outside environment. The cornea is an important mechanical and chemical barrier, and its main function is to protect the intraocular tissues of the eye. A schematic representation of the structure of the cornea is shown in Figure 1.

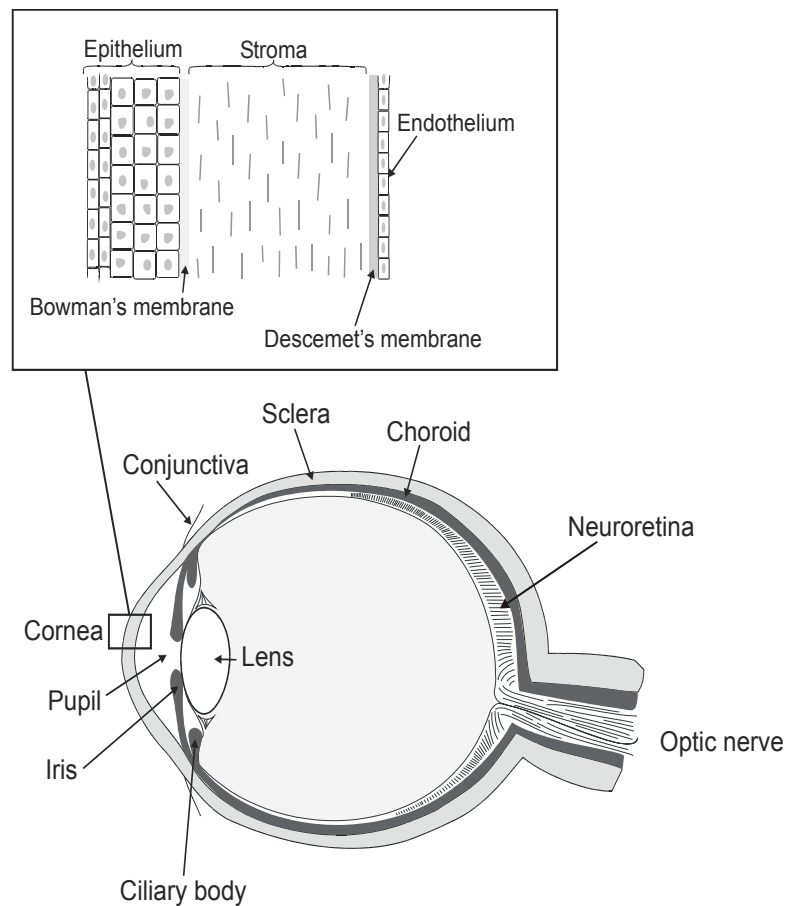


Figure 1. Anatomy of the eye and the cornea.

The transparent, avascular human cornea has a diameter of about 12 mm and it is approximately 0.5 mm thick. The cornea consists of five layers: the surface epithelium, Bowman's membrane, the stroma which forms the major part of the cornea, Descemet's membrane, and the endothelium (6;7). The corneal epithelium has a rich nerve supply and it consists 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 outside of the corneal epithelium is moistened by tear film. Beneath the corneal epithelium lies the Bowman's membrane, a resistant, acellular collagen structure. The corneal stroma forms 85-90% of the thickness of the entire cornea. It is made of regularly arranged collagen fibrils, which are responsible for corneal transparency. The collagen matrix contains keratocytes, fibroblast-like cells which produce substances essential for the maintenance of the homeostasis of the cornea. The non-cellular Descemet's membrane, secreted by the cells of the corneal endothelium, is located between the stroma and the internal endothelium. The corneal endothelium consists of a single layer of polygonal, flattened cells. Their main role is to extract water from the stroma so that the arrangement of the collagen matrix remains regular. Corneal transparency has been found to be dependent on many factors: the rapid renewal of the epithelium, the maintenance of the integrity of its structure, the state of relative dehydration of the stroma, the absence of blood vessels, and the normal metabolic activity of keratocytes and the cells of corneal endothelium, which have a vital role in the maintenance of the transparency and the normal function of the cornea (8).

THE DRAIZE EYE IRRITATION TEST

The current Draize eye irritation test evaluates the changes observed in three tissues of the eye: the cornea, the conjunctiva, and the iris (1). Albino rabbit (*e.g.* New Zealand White rabbit) is the usual test species. A group of 3-6 animals is normally used. In the original Draize test, the lower eyelid is pulled away from the eyeball, and, depending on the test material (liquid, ointment, paste, or solid), 0.1 ml or 0.1 g of the test compound is installed in the conjunctival cul-de-sac. The materials can also be placed directly onto the cornea. The other eye is left untreated or treated with the vehicle or excipient. A topical anesthetic drug is sometimes instilled before the test agent to avoid unnecessary discomfort. A washing procedure may also be included. The evaluations of ocular lesions are generally made at 1, 4, 24, 48, and 72 hours after exposure, and, if needed, at 4, 7, and 21 days (9). Several grading systems have been proposed, but the original Draize scoring method remains widely used. The scoring method involves weighting and summing six components of the directly observable changes on the anterior segment of the eye, including the density and area of corneal opacification, the severity of iritis, conjunctival redness, edema, and discharge. An illustrated standard guide is used to score irritancy. The eye irritation

potential is often summarized as the “Maximum Average Score” (MAS), which is obtained by averaging the weighted scores for individual animals at each time of observation (such as 4, 24 and 48 hours) and selecting the highest of these averages. In the original Draize test, the test scores can range from 0 to 110 points. From the maximum score of 110 points, 80 points (73% of the total score) can result from the severity and size of the corneal opacity, 20 points from the conjunctival irritation, and 10 points from the severity of iritis. While the weight sum scores are still in use for the safety assessment of cosmetics, the OECD, the United Nations, together with other international regulatory authorities have recently agreed on a Globally Harmonized System of Classification and Labeling of Chemicals (10). This is based on averaged single tissue observations, taking into account the reversibility of the observed effects.

There are several structural, physiological, and biochemical differences between the human and the rabbit eye. Rabbits have relatively low tear production, blink frequency, and ocular surface sensitivity (9;11). The anatomy of the rabbit eye is also different from the human eye. Rabbits have a nictitating membrane, a relatively larger corneal surface area, and a thinner cornea. The Draize test has been criticized for many reasons, such as the dosing of test materials, the methods of exposure, the subjectivity of observations and scoring, the lack of discrimination of fine response differences, and the overestimation of the human response (11-14). Also, the reproducibility of the Draize test has been found to be poor within and among laboratories (15-18). The test volume used in the original Draize eye test (0.1 ml) exceeds about ten times the normal volume of fluid residing in the human eye.

Despite the criticism in terms of its scientific validity and its ethical acceptability, the Draize eye test has remained until now the worldwide accepted official government-recognized procedure for predicting the potential irritant effect of chemicals in the eye, at least for moderately and slightly irritating chemicals. With the development of alternative non-animal methods to replace the Draize eye test, the data generated by the Draize test has also been used as a “gold standard”, to which the performance of *in vitro* methods has been compared.

ALTERNATIVES TO THE DRAIZE EYE TEST

In 1998, the European Centre for the Validation of Alternative Methods (ECVAM) estimated that there are approximately 70 different alternative methods for the assessment of eye irritation potential. These methods can be divided into several categories, such as computer models based on structure-activity relationships and physicochemical parameters of the compound to be tested, tests with plants and microorganisms, cell culture methods, chorioallantoic membrane (CAM)-based assays in fertilized hen's eggs, organotypic models, and three-dimensional tissue culture models. Most of the proposed alternatives are good for classifying certain types of chemicals, though not all of the chemicals across the full range of eye irritancy. Moreover, a number of proposed alternative methods appear to be capable of distinguishing between non-irritants and severe irritants, but they are not especially good at classifying between materials of mild and moderate toxicity. The Draize eye irritation test is the most widely criticized toxicity test, and consequently, several national and international validation studies on alternatives for ocular toxicology have been organized. Six major evaluation and validation studies were carried out in 1988-1997 with the most promising ocular *in vitro* alternatives. For instance, the EC/HO study, set up by the European Commission and the British Home Office, was conducted in 1992-1995 (19). Nine of the alternative methods tested failed in validation. In 1994-1997, the European Cosmetic, Toiletry and Perfumery Industry Association carried out the COLIPA international validation study testing ten *in vitro* tests designed to take into account the lessons already learned in the EC/HO study (20). The outcome of all these validation studies was that no single test, combination of tests, or testing strategy have been found capable of replacing the Draize eye test completely, but some of the assays have been shown to be considerably promising as screens for ocular irritancy (4;5). The most developed and the most widely used alternatives are the red blood cell (RBC) assay, the agarose diffusion method, the hen's egg chorioallantoic membrane (HET-CAM) test, the chorioallantoic membrane trypan blue (CAM-TB) test, the organotypic bovine corneal opacity and permeability (BCOP) test, the isolated chicken eye (ICE) test, the isolated rabbit eye (IRE) test, and the EpiOcular™ tissue model (5). The use of *in vitro* methods as screening tests is widespread in industry, since a number of alternative methods have been found to work well in-house (21), such as shown by the porcine corneal opacity and permeability assay in the prediction of the eye irritation potential of water-soluble cosmetic ingredients (22). It has been estimated that each year thousands of new products and materials are successfully tested

worldwide in *in vitro* alternative studies, but only a small fraction of the results have been published (23). Nevertheless, validation studies have not been able to establish this satisfactorily when *in vitro* test results have been compared to the historical Draize test data (24). The main reason for this is the subjectivity of the Draize test, which provides variability in the estimation of eye irritation. It is now considered that a battery of *in vitro* tests reflecting the different mechanisms of eye irritation will be needed for the complete replacement of the multipurpose animal test (25). However, in spite of the magnitude of the research focused on eye irritation, the mechanisms involved are not yet adequately understood.

CORNEAL MODELS

Corneal models, constructed by cell culturing methods, vary from simple monolayer cultures to stratified cell cultures, to epithelium-stroma co-cultures, and to more complex tissue-engineered three-dimensional corneal equivalents (Figure 2). Toxicity has been assessed by various methods such as cell count, cell detachment, colony forming efficiency, morphological changes, nutrient transport changes, measurement of cellular protein, energy metabolism disturbances, membrane changes, and transparency, histology and cytokine evaluations of the corneal equivalent.

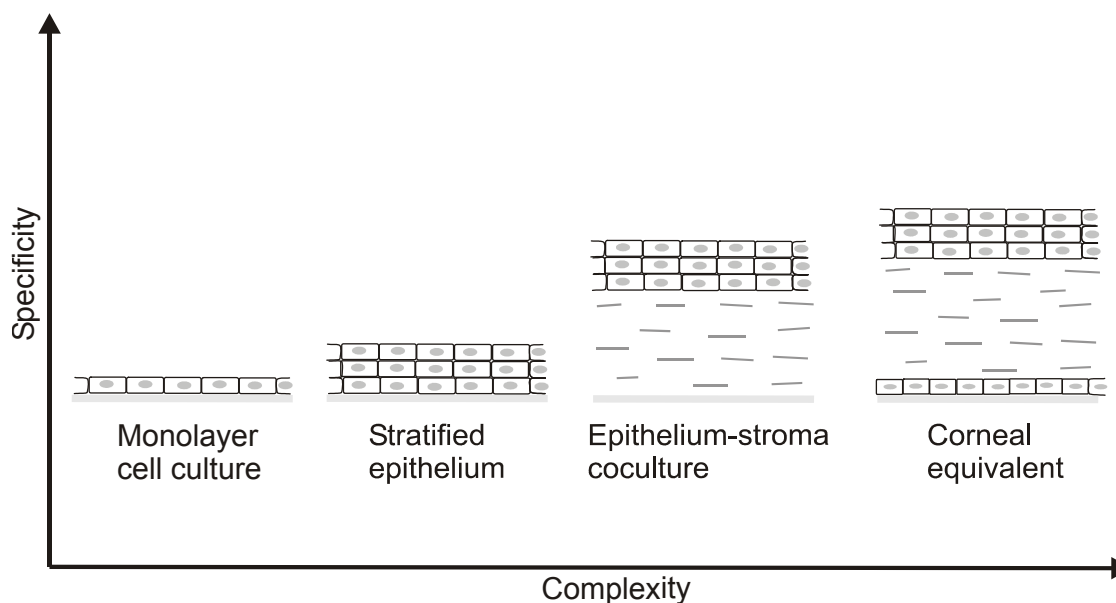


Figure 2. Corneal models constructed by cell culturing methods.

MONOLAYER CELL CULTURES

A variety of cell culture systems, both presumed target cells and non-target cells have been proposed for the assessment of ocular irritation. Monolayer cell cultures have been used with various kinds of cytotoxicity tests, which include thymidine incorporation, protein measurement by Coomassie brilliant blue, crystal violet and the Lowry reagent, MTT tetrazolium salt test, lactate dehydrogenase leakage (LDH) test, trypan blue exclusion method, propidium iodide fluorescence staining method, neutral red uptake test, neutral red release test, and fluorescence leakage test, to name only a few. Since corneal epithelial (CE) cells form the outermost layer of the eye and thus are readily exposed to injury, they have been found to be a promising tool for *in vitro* ocular toxicity screening. The use of rabbit primary CE cells in cytotoxicity testing is well known (26-33). However, the use of human primary CE cells has been limited (34-38), due to the limited availability of donor corneas. Other primary cell cultures include rabbit conjunctival cells, human skin fibroblasts, human skin keratinocytes, human buccal mucosa cells, human gingival fibroblasts, rat peritoneal cells, and isolated red blood cells from bovine, rat, rabbit, dog, and human. Today, the use of cell lines is favored, as they are more easily manageable than primary cell cultures (39). Rabbit corneal cells (SIRC, Staatens Serum Institute rabbit corneal cells) and mouse fibroblasts (Balb/c3T3) have been among the most widely used cell lines. Several non-target cell lines have also been introduced such as human dermal fibroblasts, human epidermal keratinocytes, mouse fibroblast cells (L929), Chinese hamster lung fibroblasts (V79), human erythroleukemia cells (K562), hamster kidney cells (BHK-21), Madin-Darby canine kidney (MDCK) cells, Chinese hamster ovary (CHO) cells, human hepatoma cells (Hep 2, Hep G2), and human cervical carcinoma cells (HeLa). Immortalized target cells include CE cell lines from rabbit (40-42), rat (43;44), and hamster (45). The widely used SIRC cell line has been shown to exhibit a fibroblast phenotype, which limits its value as a model for corneal epithelium (46).

The development of toxicity tests based on human corneal cell lines has been held out against the fact that to date only a few human corneal cell lines have been reported. These include the epithelial cell lines (HCE-T (10.014 pRSV-T) by Kahn *et al.* (47), HCE by Araki-Sasaki *et al.* (48), CEPI-17-CL4 by Offord *et al.* (49) and epithelial cell lines by Griffith *et al.* (50), HPV16-E6/E7 by Mohan *et al.* (51)), human keratocyte cell lines (by Griffith *et al.* (50) and HCK by

Zorn-Kruppa *et al.* (52;53)), and human endothelial cell lines (by Griffith *et al.* (50) and HENC by Bednarz *et al.* (54)). From these cell lines only corneal epithelial cell lines (HCE-T, HCE-2, and 2.040 pRSV-T) are also commercially available. The HCE cell line developed by Araki-Sasaki *et al.* (48) has been used for ocular toxicity studies as monolayer cultures (55;56), also in our laboratory (57-59). Dealing with the reliability of this kind of a simplified culture test, an important question is how well the immortalized epithelial corneal cells in culture resemble those corneal cells of the human cornea *in vivo*. We characterized the cytokeratin pattern of the HCE cell line in culture conditions that have been most often used in ocular toxicology, as pre-confluent, confluent, and post-confluent cell cultures in culture medium. The immunohistochemical characterization, by using 13 different monoclonal antibodies to cytokeratins (CKs), revealed that monolayer HCE cell cultures did not react with the monoclonal antibody AE5 and thus did not express the cornea-specific CK3 (molecular mass 64 kDa). Stratified confluent and post-confluent HCE cell cultures in medium did express CK3, but also CKs 7, 8, 18, and 19, which are typical for simple epithelium and not found in the normal cornea *in vivo* (60). The difference between the patterns of the cytokeratin expression in the corneal epithelium *in vivo* and in the HCE cell line grown in culture medium may be due to the SV40-immortalization process and/or to the culturing conditions used. The synthetic plastic substrate used in the study forced the cells to adjust to an artificially flat and rigid surface. However, in the authentic environment *in vivo*, CE cells form a multilayered epithelium supported by a stromal layer, a complex three-dimensional extracellular matrix (ECM) where CE cells are influenced by various complicated cell-to-cell and cell-to-extracellular matrix interactions (61). When HCE cells are grown on collagen matrix with keratocytes at the air-liquid interface to form a three-dimensional, stratified multilayer similar to that found in the normal human cornea *in vivo* (62), the cytokeratin pattern may be more like that of the normal cornea. As far as the authors know, there are no published results of this subject yet and thus the validity of this hypothesis still needs to be studied.

STRATIFIED EPITHELIUM

In vitro corneal models have been reconstituted by using various three-dimensional cell culture systems. In the simplest models, stratified epithelium has been cultured by using readily available

transformed dermal keratinocytes (63) or immortalized human corneal epithelial cells (64-67) at the air-liquid interface on different filter insert materials. On many applications, polyester and polycarbonate filters with or without collagen (type I or III) coating have been used. Collagen coating promotes cell attachment and stimulates cell proliferation and differentiation. As the *in vivo* corneal epithelial cells are located at the air-liquid interface, the exposure of epithelial cells to the air-liquid interface has been found to be critical also for corneal epithelial cells *in vitro*. The HCE-T model is based on the HCE-T cell line grown at the air-liquid interface on collagen membrane in serum-free medium (64-66). Studies with the HCE-T model include toxicity evaluation with transepithelial permeability to sodium fluorescence and transepithelial electrical resistance (64;65), and cell viability assessment using the MTT, Alamar Blue™, and lactate assays (66).

To date, a couple of corneal epithelium models are also commercially available. The cornea-like EpiOcular™ tissue model (MatTek Corp. Ashland, MA, USA) consists of primary human-derived epidermal keratinocytes, cultured in serum-free medium on non-coated polycarbonate cell culture inserts and differentiating to form a multi-layered structure which closely parallels corneal epithelium (68). EpiOcular has been used in combination with several cytotoxicity tests (MTT assay, LDH, IL-1 α , PGE₂, and sodium fluorescence measurements). Comparisons with the *in vivo* animal data have also been carried out, by using the ET50 value (effective time of exposure to reduce viability to 50% determined with the MTT test). The EpiOcular model is used by contract research laboratories and by industrial users in the cosmetic, personal care, household, and industrial chemical industries (69). A reconstituted corneal epithelium model (HCE™) consisting of immortalized human corneal epithelial cells grown at the air-liquid interface in chemically-defined medium on polycarbonate inserts is being marketed by SkinEthic Laboratories (Nice, France) (63;70). Possible endpoint measurements are various: tissue viability using the MTT and LDH release assays, histology, quantification of cytokine release (for example, IL-1 α , IL-6, IL-8, PGE₂), and gene expression. The HCE model is used by several cosmetic, chemical, and pharmaceutical companies to test both finished products and raw materials (69). Another stratified corneal epithelial model system constructed from transfected human corneal epithelial cells on polyester Transwell inserts coated with rat tail collagen is about to be marketed by Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA).

EPITHELIUM-STROMA CO-CULTURES

The simplest corneal equivalents consist of a stromal analogue composed of keratocytes in a collagen matrix and covered with epithelial cells (62;71-73). In the corneal model by Parnigotto *et al.*, bovine primary epithelial cells were first seeded on a feeder monolayer of bovine primary keratocytes (71). For the three-dimensional corneal model in 35-mm Petri dishes, keratocytes were mixed with collagen extracted from rat tails and epithelial cells from the feeder layer of primary cultures were seeded on the top of the keratocyte-collagen layer and grown for 7 days prior to exposure to test surfactants. Immunohistochemical analysis with the monoclonal antibody AE5, demonstrated that after 7 days of culture the *in vitro* cornea expressed CK3, the 64 kDa keratin, the known marker of corneal epithelium differentiation. For toxicity assessment, the MTT mitochondrial reduction cytotoxicity assay was applied. In the study, the sensitivity of the reconstructed corneal model to the tested surfactants was similar to that of epithelial cells, but higher than that of keratocyte cultures.

In another epithelium-stroma co-culture model by Germain *et al.*, primary cells from human origin were used (72;73). Epithelial cells, obtained by dispase digestion, were seeded with murine 3T3 fibroblasts. Keratocytes were obtained from a mixed culture of keratocytes and epithelial cells by culturing with fibroblast medium. Stromal layer was constructed from collagen (bovine or human) mixed with keratocytes and poured into a 35-mm Petri dish containing an anchorage filter paper. At 4 days of culture, three-dimensional collagen matrix containing keratocytes was seeded with corneal epithelial cells. By the third day of culture, the epithelium had 4-5 cell layers and basal layers had their characteristic cuboidal shape. After 3 days of culture, laminin staining was present in the cytoplasm of the basal cells and scatteredly at the basement membrane. Type VII collagen labeling was observed mainly in the basal cells. Fibronectin staining was obtained mostly at the epithelium-stroma junction, but also in the collagen matrix. Integrin stainings were mostly detected at the epithelium-stroma junction. In the reconstructed corneas and in the normal corneas *in vivo*, anti-integrin β_1 antibody strongly reacted with the basal side of the basal cells and less intensively with the cell membrane of suprabasal epithelial cells. Positive integrin α_3 , α_5 and α_6 stainings were also observed around the basal cells and more slightly in the suprabasal cells.

In the co-culture model by Orwin and Hubel, human primary cells and fibrillar collagen sponge (from bovine type I dermal collagen) instead of collagen gel was employed (62). The epithelial/endothelial co-culture experiments were conducted by two different methods: corneal endothelial and epithelial cells were seeded on opposite sides of the same collagen sponge, or endothelial cells were separated from the epithelial cells on the sponge by Transwell cell culture inserts with polyester membrane. Epithelial/keratocyte cultures were constructed by seeding keratocytes on the porous side of a hydrated sponge. On the 4th day of culture, epithelial cells were seeded on one surface of the sponge. In the epithelial/endothelial co-culture experiments, histological sections with hematoxylin and eosin showed a progressive migration of epithelial cells from small groups of cells near the center of the sponge on day 1 to three to four cell layers covering the sponge surface by day 14. In another set of experiments, where epithelial cells cultured on a collagen sponge were co-cultured but then separated from endothelial cells by a Transwell culture insert, histological sections showed similar results. In the epithelial/keratocyte cultures, histological sections stained with hematoxylin and eosin showed that keratocytes had infiltrated the matrix, and epithelial cells formed a continuous single layer on the top of the sponge surface. Sections stained with the monoclonal antibody AE5 against CK3 showed that all epithelial cells on the surface of the sponge had differentiated by day 15 in culture.

In the HCE model by Toropainen *et al.*, immortalized HCE cells (48) were grown on filters with various filter materials and coating procedures (67;74). In the optimal case, HCE cells were grown on polyester filters coated with rat tail collagen gel containing mouse fibroblasts (Balb/3T3). In the study, transepithelial electrical resistance and transmission electron microscopy were used. Permeabilities of H-3-mannitol and 6-carboxyfluorescein were determined to evaluate the intercellular spaces and the paracellular transport of the epithelium. Rhodamine B was used as a lipophilic marker of transcellular permeability. The transepithelial electrical resistance, morphology, and permeability of this HCE model were reported to resemble the normal intact cornea *in vivo*.

CORNEAL EQUIVALENTS

A reliable corneal equivalent mimicking the entire human cornea *in vivo*, the outer multilayered corneal epithelium, the stromal layer with keratocytes and the inner monolayer endothelium, is a good model for a more detailed study of the various cell-matrix and cell-to-cell contacts and interactions that are also present *in vivo*. To date, a few corneal models mimicking the entire cornea have been developed. Organotypic corneal equivalents are constructed on cell culture inserts step-by-step (Figure 3). Stromal keratocytes are embedded into a collagen matrix with an underlying layer of endothelial cells, and covered with multilayered corneal epithelial cells.

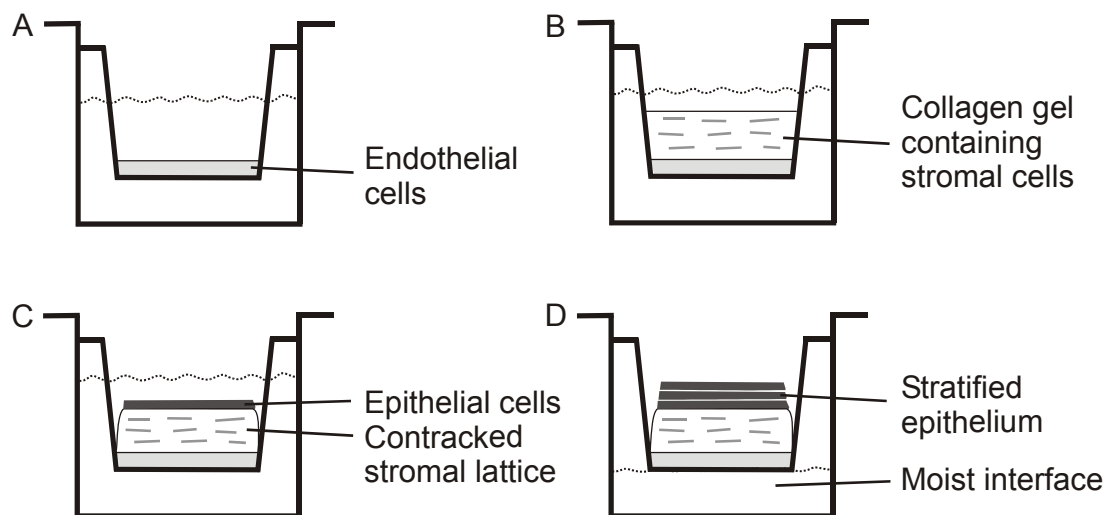


Figure 3. Diagrammatic presentation of a corneal equivalent culture. (A) Endothelial cells are seeded into a culture insert and grown to confluence. (B) A collagen layer containing stromal cells is cast on the top of the endothelial cell layer and allowed to contract submerged in medium. (C) Epithelial cells are seeded on the top of the collagen-stromal layer. (D) The culture is exposed to air-liquid interface to construct stratified epithelium.

Three-dimensional corneal equivalents have been constructed with primary bovine corneal cells (75-77), primary rabbit corneal epithelial and endothelial cells, immortalized mouse corneal endothelial cells (78), primary corneal pig cells (79-82), primary human corneal epithelial cells and fibroblasts, immortalized human endothelial cells (83), and completely with immortalized

human corneal cell lines (50;53;84;85). The first organotypic corneal equivalent was reconstructed by Minami *et al.* using primary cultured bovine corneal cells (75). Isolated keratocytes were mixed with collagen gel mixture and poured into a dish covered with nitrocellulose membrane to form a stromal layer. The culture dish was placed upside down in a larger outer dish. Endothelial layer was reconstructed by seeding endothelial cells on the coating surface of type I collagen gel on the surface of the reserved nitrocellulose membrane of the inner dish. Within 2 days, after the endothelial cells adhered well, the inner dish was reversed to its regular position in the outer dish. Isolated epithelial cells were seeded on the gel of the stromal layer. At the sub-confluent stage, epithelial cells were exposed to an air-liquid interface. The medium in the inner dish was withdrawn so the cells would be completely exposed to air. The medium in the outer dish was then withdrawn to the same level of the cultured body, so that the medium would not seep into the inner dish. The keratocytes in collagen gel matrix became elongated and remained spindle-shaped after 10 days of culture. The endothelial cells grew well for 3-7 days on the coating layer of nitrocellulose membrane. These cells were hexagon- or polygon-shaped at the confluent stage. The epithelial cells became sub-confluent within 7-10 days. At air-liquid interface, epithelial cells formed two to three cell layers in 2-5 days and five to six cell layers in 7-21 days. Electron microscopic examination showed that the epithelial cells formed a layer of stratified cells, and there were distinct desmosomes occasionally between them. The epithelial cells consisted of basal cells, wing cells, and superficial cells. All cells of the epithelial layer were positively immunostained with the cornea-specific keratin antibody AE5.

In the second corneal equivalent model, published by Zieske *et al.*, rabbit primary corneal epithelial cells were cultured over rabbit primary stromal fibroblasts in collagen matrix with or without an underlying layer of immortalized mouse corneal endothelial cells (MCEC) (78). When all three cell layers were cultured at a moist interface, hemidesmosomes, anchoring fibrils, and a continuous basement membrane were observed 2 weeks after lifting the cultures to air-liquid interface. Distribution of α -enolase (a marker for undifferentiated corneal epithelial cells) and CK3 was similar to patterns observed in the limbal region of the cornea.

In the bovine corneal model by Tegtmeyer *et al.*, primary bovine endothelial cells were seeded onto a polycarbonate filter Transwell culture insert with an underlying layer of collagen gel type I

and grown to confluence (76;77). A collagen layer containing bovine primary stromal cells was cast on the top of the confluent endothelial layer. When gel contraction was finished, bovine epithelial cells were seeded on the top of the stromal layer and grown to confluence, after which the culture was exposed to air-liquid interface for two weeks. This artificial bovine cornea was shown to exhibit a similar morphology with the excised bovine cornea (77). The immunohistochemical analyses also showed that the *in vitro* cornea was comparable to excised cornea with respect of the expression of CK3.

Schneider *et al.* have used primary corneal cells from fetal pigs for their corneal models (79;80). For cytotoxicity testing *in vitro*, corneas were constituted in microtiter plates (79). Toxicity was assessed by the using the EZ4U-system, a modified MTT test. In another cornea model from fetal pig corneal cells by Schneider *et al.*, *in vitro* corneas were constructed into polyester inserts in 6-well culture trays (80). In a porcine organotypic cornea construct by Reichl *et al.*, artificial cornea was constructed from porcine primary cells in polycarbonate Transwell inserts (81;82).

In another corneal equivalent model by Zieske *et al.*, primary human corneal epithelial cells, fibroblasts, and immortalized mouse endothelial cells (MCEC) were used (83). In the human corneal equivalent primarily based on human cells, Reich *et al.* used immortalized human endothelial cells (HENC)(54), epithelial cells (CEPI-17-CL4)(49) and primary stromal cells (fibroblasts) (84;85). In 1999, the first human corneal equivalent entirely based on immortalized human corneal cell lines was reported (50). Several different immortalization techniques were used. Most of the cell lines were immortalized by infection with an amphotropic recombinant retrovirus containing HPV16 genes E6 and E7, others were immortalized by transfection with mammalian expression vectors containing genes encoding SV40 large T antigen, pSV3neo and adenovirus E1 A 12S, separately or in combination. Another human corneal equivalent by Zorn-Kruppa *et al.* (53;87) is entirely based on SV40-immortalized human corneal cell lines, the corneal epithelial HCE cell line (48), the corneal endothelial HCEC (also known as HENC) cell line (54), and the recently developed human corneal keratocytes (HCK) (53). For cytotoxicity testing, simultaneous staining with calcein AM and ethidium homodimer-1 was used to provide live and dead probes (87). For quantification, the group has developed image processing tools to

evaluate digital images obtained from confocal fluorescence scanning microscopy measurements.

CONCLUSION

The development of an *in vitro* human corneal model for toxicity testing is well argued, because excised human corneas are not easily available for this purpose. The most widely used alternative tests to the Draize eye test are neither organ- nor species-specific. Ocular toxicology and the development of corneal equivalents have long suffered from the lack of human based immortalized corneal cell lines. The development of a number of human CE cell lines has been reported (47-51), but the development of other human corneal cell types seems to have been less successful. Only a few human corneal endothelial cell lines (50;54) and human stromal keratocyte cell lines (50;53) have been reported. The use of immortalized cell lines is also well argued, not only due to the short life span of primary cultures but also to the limited availability of human corneas. The use of cell lines also ensures better reproducibility and less product variation (53).

The three-dimensional corneal equivalent can be extensively applied for studying the mechanisms of various corneal responses in toxicology, pharmacology, corneal diseases, and ultimately in transplantation. The interest for three-dimensional human corneal models is continuously increasing, since

- compared to the Draize test an *in vitro* model based on human cells is expected to approximate better the range of species-specific cellular targets and responses to toxic injury that occur in the human eye *in vivo*,
- three-dimensional corneal model is a more sensitive and specific test system than the conventional animal test to detect the slight irritation potential of chemicals and products such as drugs and drug releasing materials,
- especially the corneal wound healing and recovery can be studied more accurately in three-dimensional corneal models than in corneal epithelial cell cultures, as the cell-matrix interactions and the cell-to-cell contacts are closely modeled,

- corneal equivalents are suitable models to study the permeability of topically applied ocular drugs,
- tissue engineered corneal equivalents may also have potential to be used as transplants in the future.

The combination of simple cytotoxicity tests based on the reflection of the mechanisms of eye injury with the more complex human corneal equivalents analyzed by using biochemical and histochemical endpoints could be an alternative to replace the Draize eye test completely.

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