Construction of a Complete Rabbit Cornea Substitute Using a Fibrin-Agarose Scaffold

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Purpose. To construct a full-thickness biological substitute of the rabbit cornea by tissue engineering.

Methods. Ten rabbit corneas were surgically excised, and the three main cell types of the cornea (epithelial, stromal, and endothelial cells) were cultured. Genetic profiling of the cultured cells was performed by RT-PCR for the genes *COL8* and *KRT12*. To develop an organotypic rabbit cornea equivalent, we used a sequential culture technique on porous culture inserts. First, endothelial cells were seeded on the base of the inserts. Then, a stroma substitute made of cultured keratocytes entrapped in a gel of human fibrin and 0.1% agarose was developed. Finally, cultured corneal epithelial cells were grown on the surface of the scaffold. Stratification of the epithelial cell layer was promoted by using an air–liquid culture technique. Corneal substitutes were analyzed by light and electron microscopy.

Results. All three types of corneal cells were efficiently cultured in the laboratory, expanded, and used to construct a full-thickness cornea substitute. Gene expression analyses confirmed that cultured endothelial cells expressed the *COL8* gene, whereas epithelial cells expressed *KRT12*. Microscopic evaluation of the cornea substitutes demonstrated that epithelial cells tended to form a normal stratified layer and that stromal keratocytes proliferated rapidly in the stromal substitute. The endothelial monolayer exhibited a pattern similar to a normal corneal endothelium.

Conclusions. These findings suggest that development of a full-thickness rabbit cornea model is possible in the laboratory and may open new avenues for research. (*Invest Ophthalmol Vis Sci.* 2006;47:3311–3317) DOI:10.1167/iovs.05-1647

The cornea is a transparent tissue in the eye that is responsible for the refraction of incoming light. The human cornea is a multilayered tissue made up of three major cell layers: the epithelium, the stroma, and the endothelium.[1] The inner layer, called the endothelium, consists of a single stratum of specialized endothelial cells.[2] The stromal layer, which accounts for 90% of the corneal thickness, is composed of stromal cells (keratocytes) contained within a matrix of collagen and protein-polysaccharide complexes. The outer layer is made of stratified corneal epithelium that plays an important role as a protective barrier.[1,3]

The cornea can be damaged by various diseases, traumas, and injuries that can cause visual impairment and even blindness.[4] So far, the only treatment for irreversible corneal damage is corneal transplantation. However, alternative solutions to corneal transplantation are needed due to the shortage of donor organs and to the complications resulting from the procedure, especially rejection of the transplanted cornea.[5]

Construction of artificial organs by tissue engineering is one of the fields that has experienced major progress during the past few years.[6] By using tissue-engineering techniques, several research groups have tried to develop an efficient substitute for the cornea that would overcome the present disadvantages of heterologous corneal transplantation.[7–9] Some researchers have proposed different tissue-engineering techniques to construct a cornea of animal origin, as the first step in the development of a human corneal substitute.[10–12] However, although some scientists developed artificial substitutes that partially mimic the human cornea,[3] a full-thickness, functional human corneal construct has not been achieved to date. Most corneal substitutes use three-dimensional coculture of the three corneal cell types and biomaterials.[5]

Different biomaterials have been used as stromal substitutes. Ideally, a good biomaterial should be biocompatible, transparent, and consistent, and corneal cells should be able to adhere and grow on it. The materials that have been used most often to construct corneal substitutes are type I collagen and fibrin. Type I collagen has been extensively used as a scaffold in tissue engineering for the construction of artificial skin, oral mucosa, corneas, and other tissues.[1,3,13–15] However, collagen is an expensive material and tends to shrink and lose volume when the cells are seeded inside the scaffold.[16,17] In addition, stromal substitutes made of collagen are not stable and are quickly degraded.[18] Human fibrin has been proposed as a stromal substitute to construct different tissue substitutes, especially human skin,[13,14] and has the advantages of low price, availability and good tolerance to cells. In contrast with collagen gels, fibrin gels are not contracted by stromal cells.[13,14] Other materials such as agarose are less commonly used in tissue engineering of the cornea due to the reduced growth rate shown by cells grown on these hydrogels.[19]

Numerous cell culture models resembling the different ocular barriers (cornea, conjunctiva, blood–retinal barrier) have been assayed during the past few years.[20] The improvement of strategies to overcome these barriers for the targeted delivery of drugs to the eye remains a major challenge. The development of corneal substitutes constructed in vitro has allowed researchers to conduct pharmaceutical testing and biomedical research including drug permeation studies,[1,17] investigation of pathologic ocular conditions, toxicological screening of compounds, and even cryopreservation assays.[21] These cornea models have been used in the study of drug transport into ocular tissues, and for other purposes, such as a potential alternative to in vivo toxicity tests, which obviates the need to

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Supported by Grants FIS 03/0141 and FIS 04/1983 from the Spanish National Ministry of Health (Instituto de Salud Carlos III).

Submitted for publication December 25, 2005; revised March 27, 2006; accepted June 9, 2006.

Disclosure: **M. Alaminos**, None; **M. Del Carmen Sánchez-Quevedo**, None; **J.I. Muñoz-Ávila**, None; **D. Serrano**, None; **S. Medialdea**, None; **I. Carreras**, None; **A. Campos**, None

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use live laboratory animals.[22–24] For these reasons, corneal equivalents are considered to be promising tools in medical and pharmaceutical research.[20]

In this work, we cultured all three types of corneal cells from rabbits and used them to develop an organotypic substitute of the rabbit cornea by tissue engineering. To improve the biomechanical properties of both fibrin and agarose components, we designed a scaffold made of a mixture of fibrin and agarose and used it as a stromal substitute.

Materials and Methods

Rabbit Corneas

To isolate corneal endothelial, stromal, and epithelial cells, 10 rabbit corneas were obtained from five adult New Zealand albino rabbits. All corneas were extracted from animals killed by lethal intracardiac injection of potassium chloride while under general anesthesia. Connective tissue and external muscles were removed from whole eyeballs, and corneas were rinsed with saline containing antibiotic solution (penicillin and streptomycin). Freshly obtained rabbit corneas were preserved at 4°C in RPMI culture medium supplemented with penicillin (100 U/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.25 μg/mL; Sigma-Aldrich, St. Louis, MO) until the moment of use. All corneas were processed within 6 hours after extraction.

This research was approved by the institutional experimentation committee, and all animals were treated according to the national and international rules of animal welfare, including the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation and Culture of Corneal Cells

Corneal endothelial cells were isolated from the rabbit corneas by incubation of the organs with trypsin 0.5 g/L-EDTA 0.2 g/L (Invitrogen-Gibco, Karlsruhe, Germany) for 10 minutes at 37°C, followed by mechanical dissection of Descemet's layer under the microscope. Endothelial cells were then cultured in 25-cm^2 tissue culture flasks with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich), 4 mM L-glutamine, adenine (24 mg/mL), 1% antibiotic solution (Invitrogen-Gibco, Karlsruhe, Germany) and a combination of the following growth factors: insulin (5 mg/mL), triiodothyronine (1.3 ng/mL), cholera toxin (8 ng/mL), and hydrocortisone (0.4 mg/mL). The medium was changed every 3 days.

To obtain epithelial cells, the corneal scleral rings of the rabbit corneas were dissected by cutting circumferentially, approximately 1 mm on either side of the cornea and conjunctival junction, according to published methods.[18] Split thickness limbal rings were cut into 2 mm^2, 100-μm thickness explants that were placed, epithelial side up, directly on a culture plate containing a small amount of culture medium, to allow the explants to attach to the culture surface. Six hours later, the tissue was submerged in DMEM medium supplemented with 10% FCS (Sigma-Aldrich), 4 mM L-glutamine and 1% antibiotic solution (Invitrogen-Gibco). To prevent fibroblast overgrowth and favor epithelial growth,[25] in some cases, the corneal scleral rings were cocultivated in the presence of a layer of 3T3 feeder cells previously inactivated by γ-irradiation (6000 rads).

Stromal keratocytes were isolated from corneas stripped of both endothelium and epithelium. After the corneal epithelial and endothelial cells had been dissected, the remaining stroma was cut into small pieces, and 3-mm explants were attached to plastic culture dishes to allow corneal keratocytes to grow. For this purpose, we used DMEM supplemented with 10% FCS (Sigma-Aldrich), 4 mM L-glutamine, and 1% antibiotic solution (Invitrogen-Gibco).

In all cases, cells were incubated at 37°C in 5% carbon dioxide. The culture medium was changed every 3 days, and subculture of the corneal cells was performed by using a trypsin (0.5 g/L)-EDTA (0.2 g/L)-solution at 37°C for 10 minutes. All cells used in our experiments were at passages 1 to 4.

Identification of Corneal Cells by RT-PCR

Total RNA was extracted from the cultured corneal cells (RNeasy Mini Kit; Qiagen, Valencia, CA). The quality of the RNA was assessed by optical inspection of ribosomal RNAs in agarose gels under denaturing conditions. For RT-PCR, 2 μg DNA was reverse-transcribed (SuperScript II; Invitrogen-Life Technologies, Gaithersburg, MD) and amplified with specific primers for keratin 12 (*KRT12*), 5′-GAACTGGGACTGCAGATGCTT-3′ (forward) and 5′-TTCAGGCTCTCGATCTGCATC-3′ (reverse); for type VIII collagen (*COL8*), 5′-CATGCAGAAAGGACCTGTGG-3′ (forward) and 5′-TCCTGGCTTTCCCATGCCT-3′ (reverse); and for vimentin (*VIM*), 5′-ATGCTTCTTTGGCACGTCTTGACCT-3′ (forward) and 5′-ACTGCACCTGTCTCCGGTATTCGTT-3′ (reverse). PCR essays were performed for 35 cycles (95°C for 30 seconds, 52°C/65°C for 30 seconds, and 72°C for 30 seconds) in a volume of 25 μL at a final concentration of 1.5 mM $MgCl_2$, 0.3 mM deoxynucleotide triphosphate, 0.25 mM of each primer and 2 units of *Taq* polymerase (Invitrogen-Life Technologies) in 1× reaction buffer. Annealing temperature was 52°C for *COL8* and *KRT12* and 65°C for *VIM*. Specific primers for the *GAPDH* gene (glyceraldehyde-3-phosphate dehydrogenase) were used in the same conditions as a control to ensure cDNA quality and loading accuracy. PCR-amplified products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Construction of Corneal Equivalents with a Fibrin-Agarose Stromal Substitute

Full-thickness, organotypic corneal constructs were developed with sequential culture techniques, as described in the following sections. To ensure both submerged culture and differentiation of the multilayered epithelium by growth at the air–liquid interface, bioengineered corneas were assembled in cell culture inserts (Transwell, Corning-Costar, Corning, NY) with 0.4-μm porous membranes.[1] This pore size allows the nutrients to cross the inserts but prevents migration of the cells to the other compartment.

To develop a full-thickness substitute of the rabbit cornea, we first subcultured 500,000 corneal endothelial cells directly on the porous membrane of the culture inserts. This cell density allows a single layer of endothelial cells to form on the membrane in 24 hours. After that time, a stromal matrix substitute was placed onto the endothelial cell layer.

Stromal substitutes consisted of cultured keratocytes entrapped in a gel of human fibrin and 0.1% type VII agarose. Fibrin was obtained from frozen plasma of human blood donations (kindly provided by Fernández-Montoya, Human Tissue Bank of Granada), and type VII agarose was purchased from Sigma-Aldrich. To produce a fibrin gel,[13,14] 12 mL human plasma was added to 9 mL saline and 500,000 cultured keratocytes resuspended in 2 mL DMEM with 10% FCS. To prevent gel fibrinolysis, the mixture was supplemented with 200 μL tranexamic acid (Amchafibrin; Fides Ecopharma, Valencia, Spain). Then 1 mL $CaCl_2$ 0.025 mM (Sigma-Aldrich) was added to start fibrin polymerization, followed by the addition of 1 mL of melted concentrated agarose (2.5% in PBS). The final concentration of agarose in the stromal substitute was 0.1%. Finally 1 mL of the mixture was seeded onto the endothelial cell layer grown in each culture insert and allowed to solidify at 37°C for 2 hours.

Twenty-four hours after the stromal substitute had solidified, rabbit epithelial cells were seeded on top of the constructed stroma (500,000 epithelial cells per construct), and cultured for 2 weeks submerged in culture medium (Fig. 1). When epithelial cells reached confluence, the air–liquid culture technique was used for two more weeks.

Microscopic Evaluation of Corneal Substitutes

For light microscopy, native, and bioengineered corneas were fixed in 4% formaldehyde, dehydrated in ethanol, and embedded in paraffin. Cross-sections 4 μm thick were cut, stained in toluidine blue and examined with a light microscope.

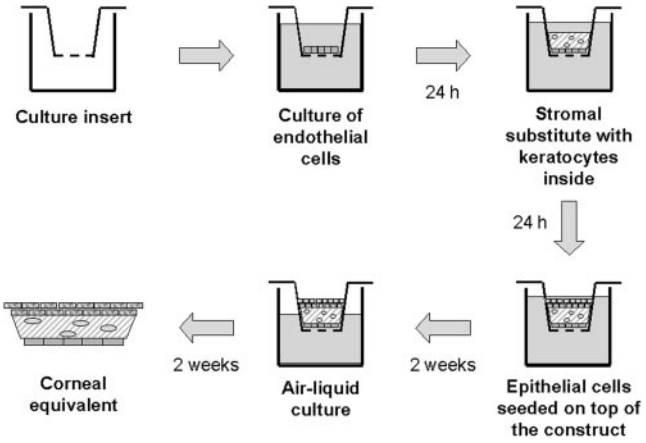

FIGURE 1. Serial construction of rabbit corneal substitutes using porous culture inserts. First, corneal endothelial cells are seeded in direct contact with the porous membrane. Then, a fibrin-agarose stromal substitute with keratocytes embedded is constructed on the endothelial layer. Finally, epithelial cells are seeded on top. The air–liquid culture technique is used to promote epithelial stratification and full formation of the corneal equivalent.

Samples for scanning electron microscopy were fixed in cacodylate-buffered 3% glutaraldehyde and postfixed in 1% osmium tetroxide for 90 minutes. After fixation, the samples were dehydrated in increasing concentrations of acetone (30%, 50%, 70%, 95%, and 100%), critical-point dried, and gold sputter-coated according to routine procedures.[26] Specimens were analyzed in a scanning electron microscope (Quanta 200; FEI, Eindhoven, The Netherlands), using a high vacuum mode.

For transmission scanning microscopy, samples fixed, postfixed, and dehydrated as described before for scanning electron microscopy were embedded in Spurr's resin and cut into ultrathin sections with an ultramicrotome. For analysis the sections were stained with aqueous uranyl acetate and lead citrate and examined with a transmission electron microscope (EM902; Carl Zeiss Meditec, Inc., Oberkochen, Germany).

Immunocytochemistry

The expression of the proteins vimentin (a marker of stromal cells)[27] and keratin 3 (overexpressed in normal corneal epithelial cells)[27,28] was assayed by immunohistochemistry in paraffin-embedded tissue sections of control and constructed corneas. Mouse monoclonal antibody against vimentin (Sigma-Aldrich) was used at 1:200 dilution, and mouse monoclonal antikeratin 3 antibody (ICN Pharmaceuticals, Basingstoke, UK) was diluted 1:100. Briefly, paraffin was removed from the slides with xylene, and the samples were then rehydrated. Slides were rinsed three times in PBS and incubated in 0.01 M citrate buffer (pH 6.0) at 98°C for 5 minutes for antigen retrieval. Samples were rinsed three times in 0.5% Triton X-100 in PBS for 5 minutes and incubated with 2% bovine serum albumin and 1% normal horse serum in PBS for 30 minutes. Primary antibodies were applied, and the tissues were incubated in a moist chamber for 1 hour at room temperature. After three rinses in 0.5% Triton X-100 in PBS for 5 minutes each, the biotin-conjugated anti-mouse IgG secondary antibody was applied, and tissues were incubated in a moist chamber for 30 minutes. Samples were then rinsed three times in 0.5% Triton X-100 in PBS and a horseradish peroxidase–conjugated streptavidin solution was applied for 40 minutes. After the sections were washed in 0.5% Triton X-100 in PBS, the color was developed with a commercial diaminobenzidine (DAB) kit (Vector Laboratories, Burlingame, CA). Samples were then counterstained in Mayer's hematoxylin and mounted on coverslips for optical evaluation. As positive controls, sections of normal rabbit cornea were used in all cases. For the negative control normal horse serum was used instead of the primary antibody.

Statistical Analysis

Cell growth rates with different cell culture techniques were compared with Student's *t*-test. To compare statistical associations between different percentages (i.e., the percentage of cultures with or without stromal contamination), we used the Fisher exact test. For individual tests, a two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Corneal Cells

Native rabbit corneal epithelial cells and keratocytes were obtained by a standard outgrowth technique from tissue explants, using culture medium without growth factors. Stromal cells displayed an elongated spindlelike shape (Fig. 2A) and showed a good growth rate in culture. Keratocytes quickly migrated from the explants and reached confluence around the ninth day of culture (9.12 ± 2.21 days). Gene expression analyses out by RT-PCR demonstrated that corneal keratocytes did not express specific RNA for type VIII collagen and keratin 12 genes, but expressed high amounts of the transcript corresponding to vimentin (Fig. 3).

In contrast, cultured corneal epithelial cells exhibited typical cobblestone morphology (Fig. 2B). In the absence of 3T3 feeder layers, corneal epithelial cells tended to migrate from the corneal explant around the fifth day of culture (5.1 ± 2.4 days), reaching confluence and forming a monolayer of cells after 11 days of culture (11.1 ± 5.6 days). However, when the feeder layer of inactivated 3T3 cells was used, corneal epithelial cells started to adhere to the culture surface around day 6 of culture (6.3 ± 2.3 days), reaching confluence in 13 days (13.6 ± 5.2 days). In contrast to previous reports of better epithelial cell growth when 3T3 cells are cocultured,[29] the differences between culture methods in our study were not statistically significant ($P > 0.05$ for the Student's *t*-test). Nevertheless, use of the feeder layer prevented stromal contamination of the epithelial culture, which was detected in only 13.3% of the cases when the 3T3 layer was used (2/15 cultures), but in 60% of cultures without the feeder layer (9/15 cultures). The differences were significant according to the Fisher exact test ($P < 0.01$). As previously reported,[27,30,31] corneal epithelial cells showed high expression of the transcripts corresponding to the pair of 3/12 cytokeratins but did not express the *VIM* and *COL8* genes by RT-PCR (Fig. 3).

We also cultured corneal endothelial cells from Descemet's membrane, isolated from the rabbit corneas with a success rate of 50% (5/10 corneas). In culture, these cells displayed morphologic similarities to native corneal endothelial cells in vivo, although the cells tended to show a polygonal, elongated shape rather than the typical hexagonal shape in the original cornea (Fig. 2C). Isolated corneal endothelial cells proliferated rapidly in culture, starting to grow from the fifth day (mean, 5.1 ± 2.3 days), and forming a cell monolayer after 12 days of culture in media supplemented with a mixture of growth factors (12.6 ± 5.4 days). As expected,[27,32–35] cultured corneal cells exhibited a relative overexpression of the *COL8* gene, whereas the *VIM* and *KRT12* genes were not expressed (Fig. 3).

Full-Thickness Corneal Equivalents

Corneal equivalents were efficiently constructed with porous culture inserts. With this system we observed attachment and growth of the corneal endothelial cells to the porous membranes, suggesting that all nutrients in the culture medium crossed the membrane to the upper compartment of the inserts. The stromal substitute we developed is composed of human fibrin and 0.1% agarose. Embedded in this scaffold,

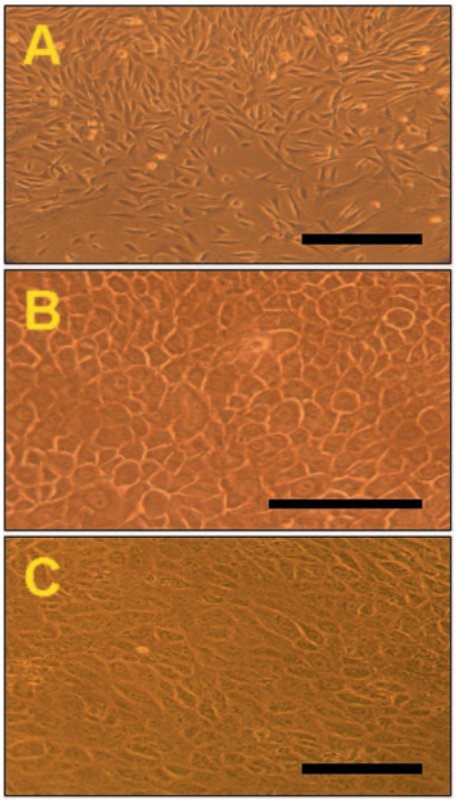

FIGURE 2. Phase-contrast microscopic image of cultured corneal cells. (**A**) Stromal keratocytes. (**B**) Corneal epithelial cells. (**C**) Corneal endothelial cells. Scale bars: 200 μm.

rabbit keratocytes showed good survival and rapid proliferation. After 1 to 3 days of culture, stromal keratocytes incorporated into the fibrin-agarose gels became elongated and spread out in the lattice (Figs. 4, 5). No contraction of the fibrin-agarose gels was observed in any of the cultures. Evaluation of normal and bioengineered corneas with scanning electron microscopy demonstrated that the constructed stromal substitutes were structurally very similar to native tissues (Fig. 5). After the epithelial cells were seeded on top of the stromal substitutes, a monolayer of cells was observed in 7 to 10 days of submerged culture. Stratification of the constructed corneal epithelium was observed after 2 weeks of exposure of epithelial cells to air in the culture inserts.

Microscopic evaluation of the corneal constructs revealed that epithelial cells tended to form a normal, stratified, tightly

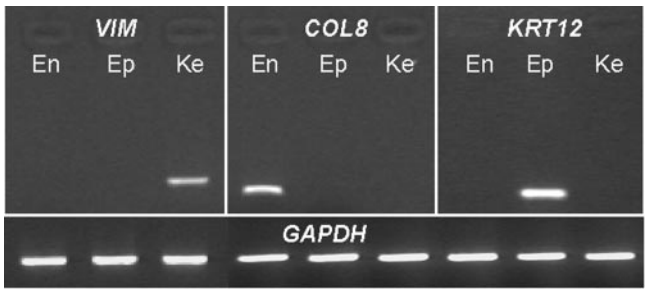

FIGURE 3. *VIM*, *COL8*, and *KRT12* mRNA expression in cultured corneal cells, as assessed by RT-PCR. Expression of the *GAPDH* gene was used as a control for RNA loading. RT-PCR analyses were conducted for cultured corneal endothelial cells (En), cultured corneal epithelial cells (Ep), and cultured stromal keratocytes (Ke).

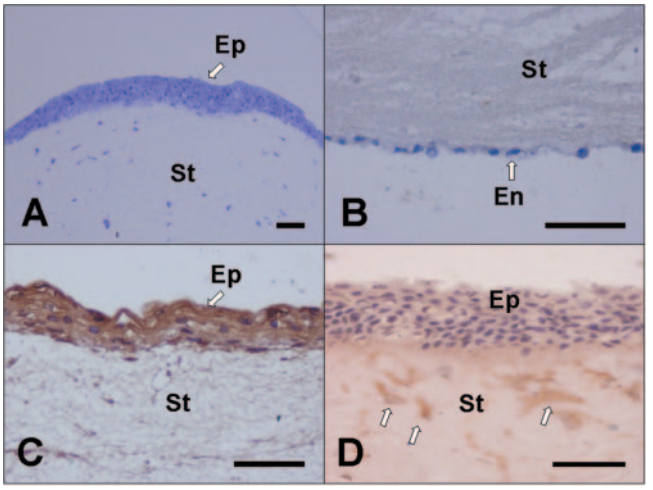

FIGURE 4. Light microscopy histologic evaluation of the rabbit cornea constructs subjected to the air–liquid culture technique for 2 weeks. (**A**) Epithelial cells (Ep) forming a tight, stratified cell layer on top of the stromal substitute (St), which contains keratocytes (toluidine blue staining). (**B**) Corneal endothelial cells (En) cultured at the bottom of the construct form a cell monolayer resembling the native corneal endothelium (toluidine blue staining). (**C**) Immunohistochemical analysis of the constructed corneas with a monoclonal antikeratin 3 antibody showed strong cytoplasmic staining of the biodeveloped epithelial cell layer (*arrow*). (**D**) Immunohistochemistry for the stromal marker vimentin was positive only for stromal keratocytes (*arrows*). Scale bar, 100 μm.

packed epithelium with several layers of cells, and that superficial cells were flattened after exposure to air (Figs. 4, 5). In addition, immunohistochemical analysis of the constructed corneas demonstrated phenotypic similarities with normal, native rabbit corneas, with high expression of cytokeratin 3 in the epithelial cell layer and expression of vimentin in the stromal cells of the construct (Fig. 4). These findings suggest

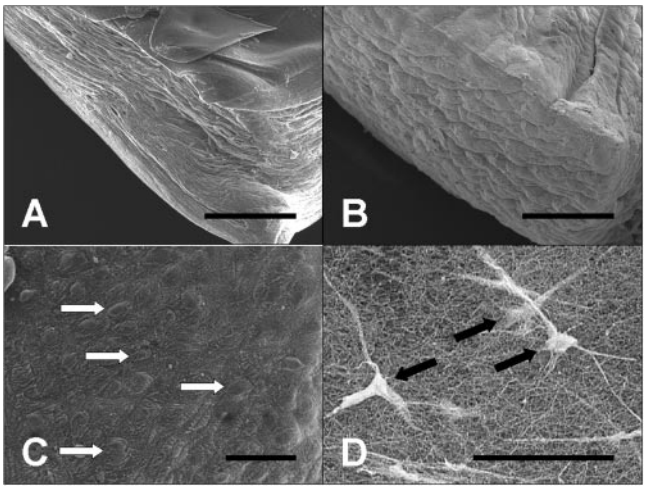

FIGURE 5. Comparative scanning electron microscopic images of native and bioengineered rabbit corneas. (**A**) Normal rabbit corneal limbus showing the typical multilayered structure of the stroma. (**B**) Constructed three-dimensional cornea made with keratocytes entrapped in a mixture of fibrin and agarose. For both (**A**) and (**B**), the *top* corresponds to the endothelial side of the cornea. (**C**) Surface of the bioengineered cornea showing a tight multilayer of flattened epithelial cells (*arrows*) after exposure of the culture to air. (**D**) Stromal cells (*arrows*) cultured within the stromal substitute tended to spread and differentiate. Scales bars: (**A**, **B**) 500 μm; (**C**, **D**) 50 μm.

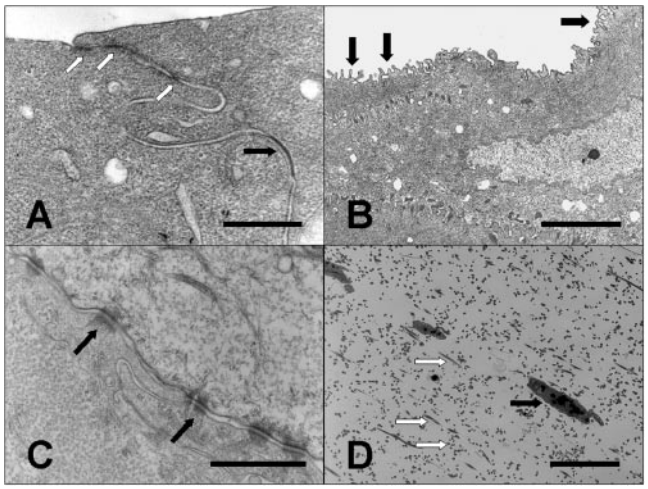

FIGURE 6. Transmission electron microscopic images of the constructed corneal epithelium. (**A**) Ultrastructure of the apical epithelial cell layer revealed the presence of at least two types of intercellular unions: tight junctions (*black arrow*) and desmosomes (*white arrows*). (**B**) Microvilli were detected on the surface of the epithelium (*black arrows*). (**C**) Numerous desmosomes are present in the epithelial cell layer (*arrows*). (**D**) Ultrastructure of the constructed stroma with intermingled keratocytes (*black arrow*) and fibrin fibers (*white arrows*). Scale bars: (**A**, **C**) 0.5 μm; (**B**) 5 μm; (**D**) 1 μm.

that both the stromal and the epithelial layer of the construct were biologically equivalent to those of native corneas. Analysis of the ultrastructure of the epithelial cell layer revealed that the most apical cells were flat with tight junctions, microvilli, and desmosomes (Fig. 6). However, if the cells were grown without the air–liquid interface, only two to three cell layers lacking flattened cells and tight junctions were formed. The macroscopic appearance of the reconstructed cornea showed that it was a translucent tissue.

DISCUSSION

Corneal transplant is the most commonly used and successful transplantation procedure performed in the United States and Europe.[36] However, the drawbacks associated with the procedure are increasing. The supply of human corneas available for transplantation, which is always limited, may be threatened by the increasing use of refractive surgery, since these corneas are not suitable for transplantation.[37] Moreover, the complications derived from the heterologous origin of corneal implants, including the risk of infection and immune rejection, make it necessary to develop new methods for corneal reconstruction from autologous corneal tissues.[9,36] In this regard, biological corneal equivalents developed by tissue engineering and synthetic keratoprostheses[38] have been proposed as substitutes for corneal transplantation to restore impaired vision. Tissue engineering of the cornea thus represents a paradigm shift in medical treatment to overcome the present disadvantages or corneal transplantation.[9] Several attempts are currently under way to develop an organotypic biological cornea substitute.[1,3,10–12,15]

In this study, we isolated and maintained in culture all three major cell types in the rabbit cornea: epithelial, stromal, and endothelial cells. Use of a feeder layer of inactivated cells has previously been associated with high proliferation rates of cultured epithelial cells.[25,29] Our results, however, showed that corneal epithelial cells grew at the same rate, with or without the feeder layer, although the coculture technique prevented fibroblast contamination from stromal cells.

In contrast to human tissues, it has been reported that endothelial cells of the rabbit cornea show a good proliferation rate in culture,[2] making these cells potentially useful in tissue engineering. Efficient culture of the three main types of corneal cells in the laboratory offers new possibilities in different fields of research, especially those related to diseases of the ocular surface, and opens the door to the construction of artificial organs by tissue engineering. However, in this milieu, it is important to identify accurately the different cell types in culture. After isolation and culturing, most cells tend to become partially undifferentiated and can be difficult to identify on the basis of morphologic features. Nevertheless, RT-PCR gene expression analysis is highly cell-type–specific, and good markers are available to identify the different corneal cells.[39] In this work, RT-PCR analysis demonstrated that corneal endothelial cells kept in culture expressed high amounts of RNA specific for the gene that encoded type VIII collagen, whereas epithelial cells expressed the transcript of keratin 12, and stromal keratocytes did not express type VIII collagen or keratin 12, but showed high expression of the vimentin gene. Previous studies reported that type VIII collagen expression is restricted to Descemet's membrane of the cornea, and that the only cells expressing high levels of the *COL8* transcript are corneal endothelial cells.[27,32–35,40] In contrast, expression of the genes encoding for cytokeratins 3/12 is a well-established marker of corneal epithelial differentiation.[27,30,31,41] These and other highly specific techniques should be used to confirm the phenotype of cells to be used to construct organs by tissue engineering.

Human and animal corneal epithelial and stromal cells have been kept successfully in culture. However, in vivo and ex vivo observations, as well as tissue culture studies, suggest that the relative proliferative capacity of corneal endothelial cells may differ among species. For example, bovine, rat, and rabbit[2,42] endothelial cells grow easily in culture. However, although a few investigators have reported the successful culture of human endothelial cells, it is well known that monkey and human cells have a very low proliferation index and are thus difficult to culture.[2] Although some researchers have reported the successful culture of human endothelial cells,[43] efficient artificial models of the human cornea using normal cells of the three main cell layers have not been developed to date. Thus far, corneal constructs using human cells of the three main layers of the cornea have used SV40-transformed immortalized endothelial cells.[3] The use of genetically manipulated cells, however, is highly restricted to certain nonclinical purposes, since the proliferation index of these cells is much higher that of native cells.[44] For these reasons, a biological model of the rabbit cornea such as that reported here, holds potential as an efficient substitute for the human cornea in physiological or pharmacological studies in vitro.

Once the corneal epithelial, stromal, and endothelial cells were isolated and cultured, we used a sequential culture technique to develop an efficient substitute for the rabbit cornea. To construct organotypic corneal substitutes, we used commercially available culture inserts[1] for two reasons. First, the presence of a porous membrane at the base of each insert allows the culture of an endothelial cell layer at the bottom of the constructs without compromising the nutrient and oxygen supply to these cells. Evaluation of our corneal substitutes with scanning electron microscopy, showed that a confluent cell monolayer of endothelial cells was formed below the stromal substitutes. Second, the design of the devices allowed us to use the air–liquid culture technique to promote stratification of the uppermost layers of the corneal epithelium. Culture inserts of different types have been used to construct various types of tissue by tissue engineering[1,45–48] as an efficient way to promote epithelial stratification.[22,45]

Several corneal tissue models have been produced in vitro with animal cells. Zieske et al.[11] reported a method to develop rabbit corneal equivalents by including stromal keratocytes in a collagen gel with or without an underlying layer of immortalized mouse corneal endothelial cells. Another cornea model using the three cell types (epithelial, stromal and endothelial cells) of the bovine cornea on collagen matrices was reported by Minami et al.,[10] whereas Schneider et al.[12] developed a cornea construct by using the three cell layers of the fetal pig cornea. In all these cases, the use of collagen gels was handicapped by keratocyte-mediated contraction of the hydrogels.[7,29] In contrast, some researchers have used fibrin matrices as stromal substitutes in different tissues, including the human cornea.[25,49] However, the mechanical properties of pure fibrin polymer gels are not always comparable to those of the corneal stroma in terms of consistency and transparency.

In general, the properties of native soft tissues cannot easily be duplicated by synthetic materials. Unlike simple polymer gels, many biological materials such as the cornea stiffen as they are strained, thereby preventing large deformations that could threaten tissue integrity.[50] In this work, we synthesized a stromal substitute of keratocytes entrapped in a gel made of a mixture of fibrin and 0.1% agarose. These constructs demonstrated better consistency than fibrin alone, with good transparency, and allowed us to perform sutures on the artificial corneas. In addition, fibrin-agarose gels sustained a functional epithelial layer very similar to the normal corneal epithelium, with epithelial cells showing a good degree of attachment while growing on the matrix. Furthermore, fibrin-agarose gels did not contract as collagen gels did,[7] and their microscopic appearance was similar to that of native corneas. As reported for other reconstructed corneal epithelia[7,51] and found in our tissue cultures, the uppermost superficial cell layer of the corneal epithelium appeared flattened, with numerous desmosomes, as is also the case in normal corneas in vivo. These results suggest that the corneal epithelium that developed in our tissue-engineering model formed a tight barrier, making the constructed corneas efficient substitutes for native corneas for in vivo or in vitro purposes and pharmacological studies.[7,17,20,21,23,51] Moreover, protein expression analyses of the constructed corneas demonstrated a high expression of cytokeratin 3 in the cultured epithelium, and specific expression of vimentin restricted to stromal cells. These highly specific analyses imply that our biodeveloped corneas were equivalent to native corneas.

One of the main goals of tissue engineering of the cornea is to construct a biological equivalent of the human cornea from small fragments of corneal biopsy tissue. However, attempts to develop a full-thickness cornea equivalent from limbal explants are handicapped by the inability of corneal endothelium to grow from small pieces of corneal tissue. Our findings suggest that corneal substitutes based in fibrin–agarose matrices are similar to native rabbit corneas, resembling the ultrastructure of a normal native organ. The fibrin–agarose complexes described in this study appear to satisfy the criteria for biomaterials used in tissue engineering of the cornea: they are simple to manufacture and are likely to be well tolerated when implanted in laboratory animals. We thus believe that development of a full-thickness cornea model in laboratory is achievable. Constructs based on rabbit corneal cells would provide an ideal model for in vitro investigations of drug permeation, as excised human corneas are not available for this purpose.[17] Thus, our organotypic cornea model in rabbits is potentially useful for many in vitro assays of the biological properties of the cornea. The future challenge will be to improve the model without compromising the transparency of the constructed tissue.

Acknowledgments

The authors thank Maria Angeles Robles for technical assistance and Karen Shashok for improving the use of English in the manuscript.

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