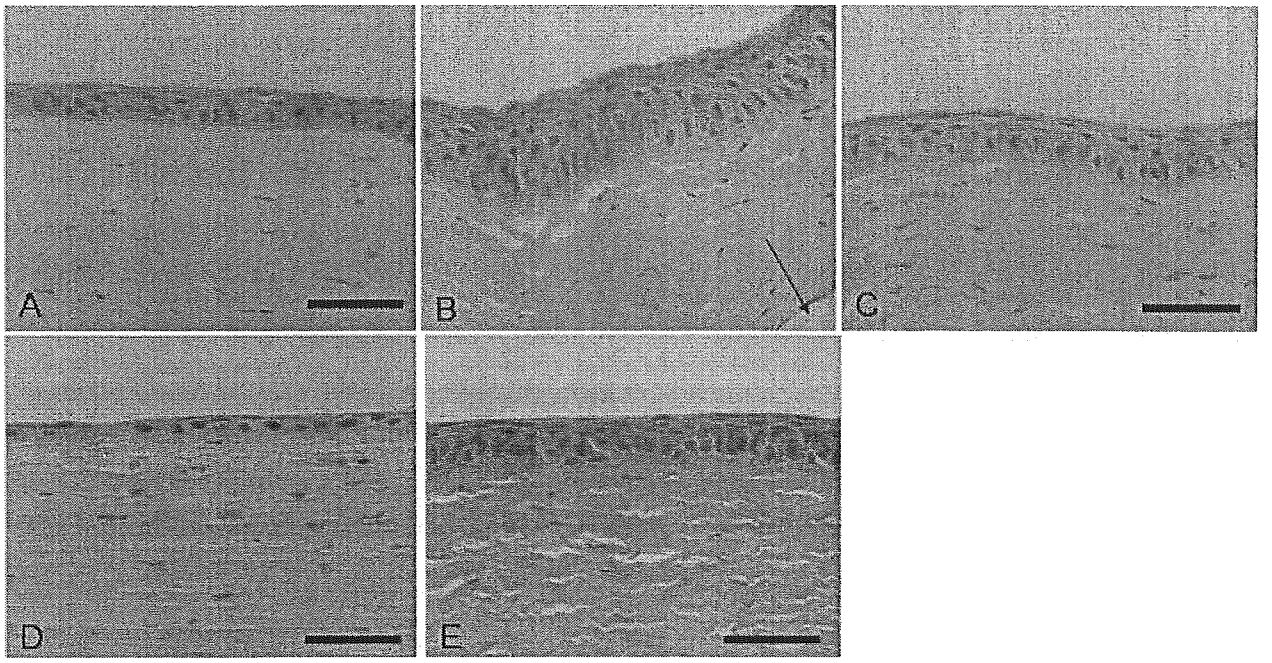

Figure 2. HE staining sections of rabbit cornea at 4 weeks after implantation. Rabbit corneal epithelium (A) above the implanted PVA-COL, (B) near the edge of PVA-COL, and (C) in the peripheral cornea. Epithelium is thin over the implanted PTFE polymer (D), compared to normal control cornea (E). Arrow indicates the space occupied by the implanted PVA polymer. Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

content. Some sections were treated with saliva at 37°C for 10 min to digest the glycogen and were used as a negative control.

Human Corneal Epithelium on PVA-COL

Air-Lift Cell Culture. PVA-COL was placed on a culture insert (Transwell clear, Corning, Corning, NY) in six-well plates, in which mitomycin C-treated 3T3 feeder cells ($2.4–5 \times 10^4/cm^2$) were seeded on the bottom. Human limbal segments, which contain corneal epithelial stem cells, were plated on the PVA-COL polymer so that the epithelium can expand onto the polymer ($n = 9$). After cells reached confluency, the volume of the medium was reduced until the epithelial cells were placed at the air–liquid interface. The culture medium used was a 1:1 mixture of Dulbecco's modified eagle medium and Ham's F12 medium (DMEM/F12, Gibco BRL, Rockville, MD) containing 15% fetal bovine serum, and supplemented with insulin (5 μg mL^{-1}, Sigma-Aldrich, St. Louis, MO), cholera toxin (10 ng mL^{-1}, EMD Biosciences, San Diego, CA), human recombinant epidermal growth factor (10 ng mL^{-1},Gibco BRL) dimethyl sulfoxide (0.5 %, Sigma-Aldrich), penicillin (0.7 mg mL^{-1},Wako Pure Chemical Industries, Osaka, Japan), and streptomycin (1.39 mg/mL, Wako). The culture medium is a modified version of the SHEM medium originally reported by Jumblatt and Neufeld.[18] Aprotinin (667 KIU, Wako) and laminin (1 mg mL^{-1}, BD Bioscience, San Jose, CA) were added to the medium during air-lift cultures.

Immunohistochemistry. Paraffin sections for immunohistochemistry were prepared according to the AMeX method.[19] Samples were fixed in acetone at 4°C overnight, immersed in methyl benzoate at RT for 1 h (×2), and immersed with xylene at RT for 1 h (×2). After incubation in 60°C paraffin for 1.5 h (×2) and overnight once; samples were embedded in paraffin. AMeX paraffin sections were dewaxed, immersed in acetone to remove xylene, and rinsed in PBS. Sections were treated with protease XXV (Neomarkers, Lab Vision corporation, Fremont, CA) at 37°C for 5–10 min for the immunostaining of collagen type IV and collagen type VII. Sections were blocked with PBS containing 10% normal donkey serum and 1% BSA, and treated with antikeratin 3/12 (AE5, 0.2 μg/mL, Progen Biotechnik GmbH, Heidelberg, German), antioccludin (clone OC-3F10, 10 μg/mL, Zymed Laboratories Inc, South San Francisco, CA), anticollagen type IV (clone IV-3A9, 10 μg/mL, Daiichi Fine Chemical Co. Ltd., Toyama, Japan), and anticollagen type VII (clone LH7.2, 1 μg/mL, Neomarkers) mouse monoclonal antibodies at 4°C overnight. After washing, sections were treated with FITC-conjugated donkey antimouse IgG antibody (28 μg/mL, Jackson ImmnoResearch Laboratories Inc, West Grove, PA) or Cy3-conjugated donkey antimouse IgG antibody (Chimicon International Inc., Temecula, CA), and counterstained with 6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg/mL, Dojindo Laboratories, Kumamoto, Japan). After coverslips were applied, fluorescence was detected by the Zeiss Axioscop 2. Filter sets used were by Carl Zeiss 488001-0000-000 (excitation filter BP365/12, dichroic mirror FT395, barrier filter LP397) for DAPI, 488009-0000-000 (BP470/40, FT510, LP520) for FITC, and 488015-0000-000 (BP546/12, FT580, LP590) for Cy3.

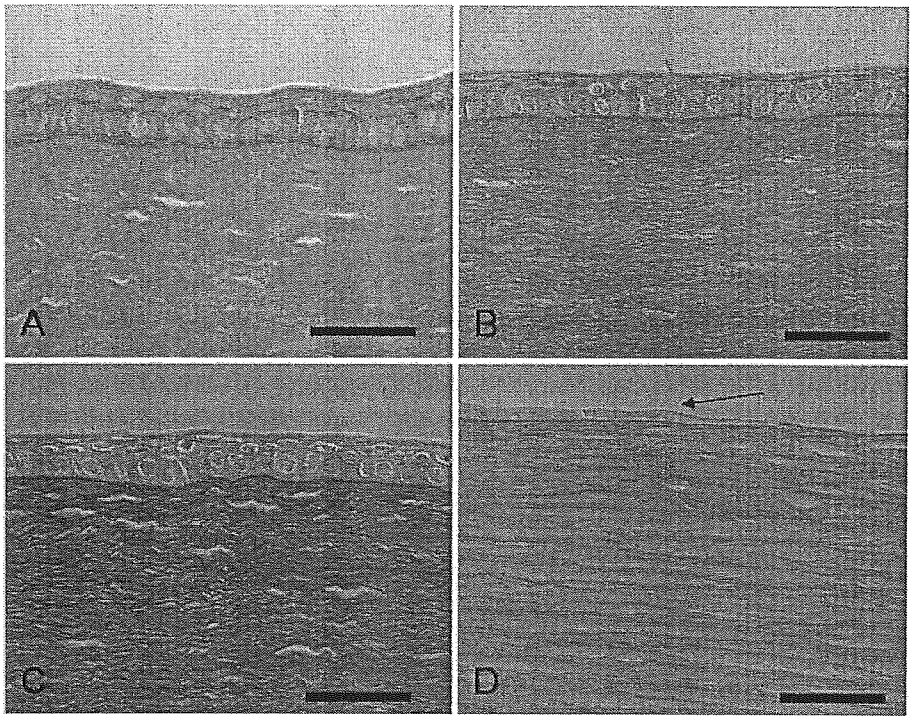

Figure 3. PAS staining sections of rabbit cornea at 4 weeks after implantation. (A) Normal corneal epithelium. (B,C) The epithelium above the implanted PVA-COL in sections (B) without or (C) with saliva treatment, which was performed to digest glycogen granules. (D) The epithelium above PTFE (arrow). Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Horseradish Peroxidase Permeability Assay. In order to observe the formation of tight junctions at the epithelial surface, the horseradish peroxidase (HRP) diffusion assay was performed as described previously.[20] In brief, medium was removed from the culture, and 200 μL of HRP in PBS (100 mg/mL, Wako Pure Chemical Industries) was incubated on the apical surface of the epithelium at RT for 45 min. Samples were fixed with 2.5% glutaraldehyde (GA) in 0.2*M* PBS at 4°C overnight, and washed twice with PBS. Samples were treated with 0.2% H_2O_2 at RT for 30 min to block endogenous peroxidase, washed with PBS twice, and treated with DAB kit (Vector Laboratories Inc., Burlingame, CA) at RT for 10 min. Samples were embedded in paraffin, or postfixed for electron microscopy.

Electron Microscopy. Specimens were fixed with 2.5% glutaraldehyde solution in 0.2*M* phosphate buffer (pH 7.4) for 4 h at 4°C and washed with 0.1*M* phosphate buffer. The sample was then postfixed in 1% osmium tetraoxide, dehydrated in a series of ethanol and propylene oxide, and embedded in epoxy resin. Semithin sections (1 μm) were stained with methylene blue. Ultrathin specimens were then sectioned with the use of a microtome (LKB; Gaithersburg, MD) with a diamond knife. Sections in the range of gray to silver were collected on 200-mesh grids, stained with uranyl acetate and lead citrate, and examined under an electron microscope (model 1200 EXII; JEOL, Tokyo, Japan).

Rabbit Corneal Epithelium on PVA-COL

Cell Culture. Rabbit epithelial cells were cultured according to the human cell culture protocol with slight modifications in initial seeding. Rabbit limbal cells were isolated from the limbus by trypsin-EDTA treatment (0.05 %, 37°C, 60 min), and seeded onto PVA-COL using glass cloning rings (n = 5). Subsequent procedures, including air-lifting, were done as described above.

Lamellar Grafts with Cultured Rabbit Epithelium in Rabbit Eyes: Suture Durability Study. PVA-COL with cultured epithelium was transplanted as a lamellar graft into the left eye of healthy rabbits (n = 3) with the us of 10-0 nylon running sutures. The surgery was done as a test of suturability of the epithelium-PVA-COL composite. All animals received topical antibiotics (levofloxacin) and steroids (betamethasone) twice a day following surgery, and were sacrificed once sutures became loose 2–3 weeks following surgery.

RESULTS

Intracorneal Implants

Epithelial Cell Layers and Glycogen Content. PVA-COL were implanted into the rabbit stroma for 4 weeks [Figure 1(A)]. Slit lamp examination did not show remark-

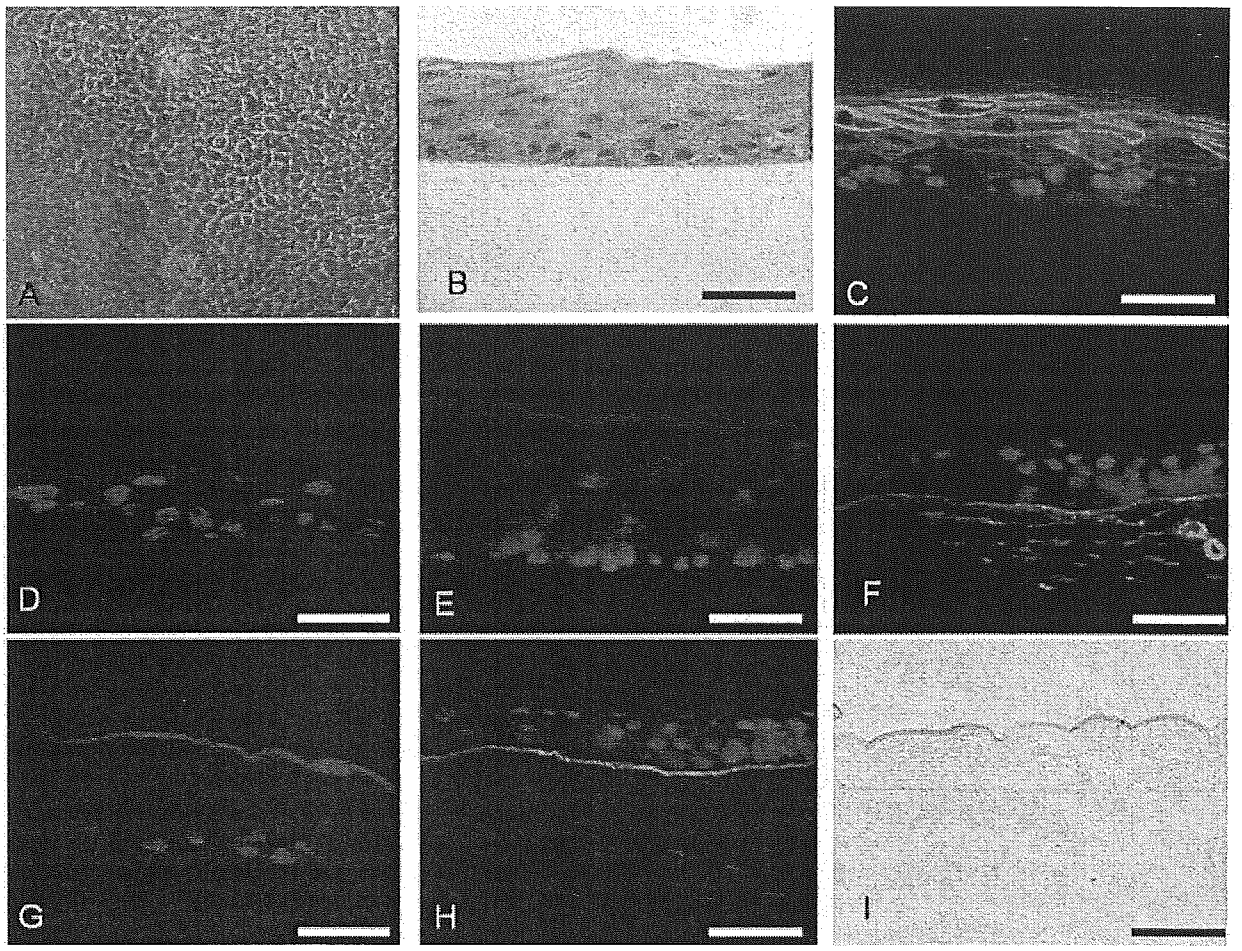

Figure 4. Human corneal epithelial cells air-lift cultured PVA-COL. (A) Phase-contact micrograph of human epithelial cells air-lift cultured for 1 week. (B) HE-stained paraffin section. (C–H) Immunohistochemistry against the corneal epithelium differentiation marker keratin 3/12 (C, green), tight-junction protein occludin (D, red), basement membrane component collagen type IV [(E) cultured corneal epithelium and (F) normal human limbus, green], and collagen type VII [(G) cultured corneal epithelium and (H) normal human limbus, green]. Nuclei were stained with DAPI (blue). (I) The epithelium after HRP permeability assay. Bars = 50 μm.

able changes 4 weeks after implantation [Figure 1(B,D)]. Fluorescein exclusion stains showed that the epithelium was intact, and that the fluorescent dye did not diffuse into the stroma [Figure 1(C,E)]. Figure 1(F) shows the cornea implanted with PTFE, which developed a defect (green fluorescence indicated by arrow) in the epithelial layer. The number of cell layers was counted in the epithelium above PVA-COL [Figure 2(A)], near the edge of PVA-COL [Figure 2(B)], and in the peripheral cornea [Figure 2(C)]. The average number of epithelial layers in each group (n = 3) was 2.91 ± 0.05, 4.63 ± 1.21 and 3.1 ± 0.56, respectively. The number of cell layers was similar to normal rabbit epithelium shown in Figure 2(E). On the other hand, the epithelium in the PFTE control cornea consisted of 0–1 layer [Figure 2(D)]. The normal rabbit epithelium was PAS positive, indicating abundant glycogen granules [Figure 3(A)], which disappears after digestion of glycogen with saliva. The epithelium over the PVA-COL showed the same staining pattern as control corneas [Figure 3(B,C)]. Epithelium remaining on the PFTE cornea was depleted of glycogen [Figure 3(D)].

Human Corneal Epithelium on PVA-COL

Air-Lift Cell Culture. Human limbal epithelial cells were cultured on PVA-COL along with mitomycin-C treated 3T3. Although 3 out of 9 trials resulted in initial epithelial detachment from PVA-COL, the others adhered to PVA-COL [Figure 4(A)] and formed a stratified epithelium [Figure 4(B)]. The stratified epithelium consisted of small round basal cells, and flattened suprabasal cells.

Immunohistochemistry. Two samples were examined with immunohistochemistry. The anti-keratin 3/12 antibody AE5, a differentiation marker of the corneal epithelium, stained suprabasal layers [Figure 4(C)]. Tight-junction–associated protein occludin was observed in the uppermost layer

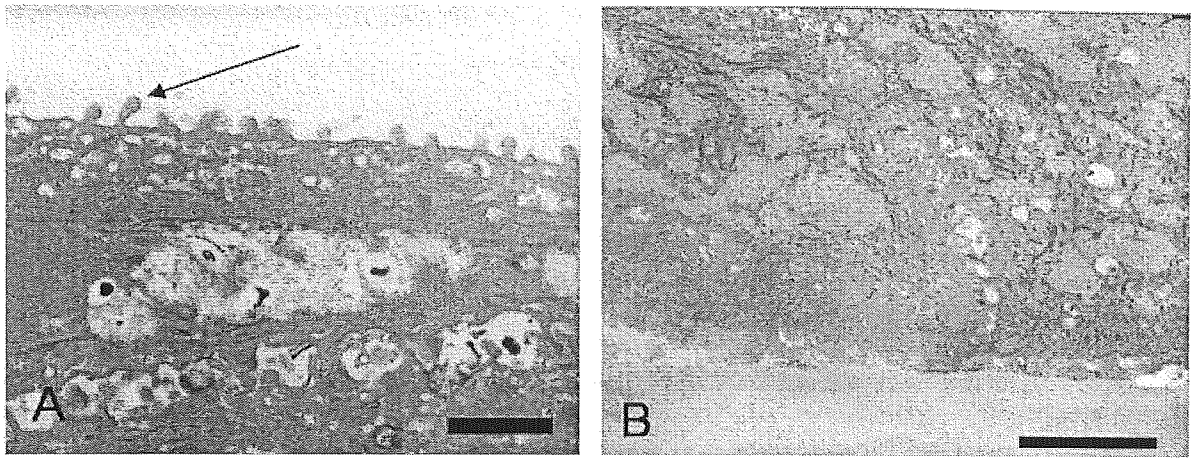

Figure 5. Electron microscopy of human corneal epithelial cells cultured on PVA-COL. (A) Superficial layers with characteristic microvilli (arrow). (B) Basal cells at the PVA-COL junction do not show basement membrane architecture. Bars (A) = 1 μm, (B) = 4 μm.

of the epithelium [Figure 4(D)]. Antibodies against basement membrane proteins did not stain with cultured human epithelium on PVA-COL [collagen type IV: Figure 4(E), collagen type VII: Figure 4(G)], although it stained normal human corneal limbus [collagen type IV: Figure 4(F), collagen type VII: Figure 4(H)].

Horseradish Peroxidase Permeability. HRP solution incubated on the surface of the stratified epithelium was not observed within the intracellular space [Figure 4(I)]. This indicates a functional tight junction at the surface of the cell sheet, which is important in the exclusion of microbes following transplantation.

Electron Microscopy. Microvilli-like structures were observed on the superficial cells [Figure 5(A)], whereas basement membrane structures were not observed at the epithelium-PVA-COL interface [Figure 5(B)], consistent with the results observed by immunohistochemistry.

Rabbit Corneal Epithelium on PVA-COL

Lamellar Grafts with Cultured Rabbit Epithelium in Rabbit Eyes: Suture Durability Study. Rabbit corneal epithelial cells were cultured and stratified on PVA-COL [Figure 6(A)], which was confirmed to be a well-differentiated epithelium expressing keratin 3/12 in the superficial layers [Figure 6(B)]. The epithelialized graft was easy to handle, flexible, and suturable, similar to a donor cornea [Figure 6(C)]. The epithelium excluded fluorescein dye, indicating that surgical trauma did not compromise the stratified epithelium [Figure 6(D)]. However, the sutures became loose after a few days due to inflammation, causing the epithelium to detach from the polymer surface.

DISCUSSION

Reports have shown that surface-modified PVA may be used as a KPro material that permits surface epithelialization.[13,15] Latkany et al. developed surface-modified PVA manipulated with radio frequency (rf) argon plasma treatment, and indicated that rabbit epithelial cells adhered and proliferated on the surface.[12] In order to support a healthy, stratified corneal epithelium for extended periods of time, the scaffold material must allow nutrients to pass from the interior of the eye. Among the various molecules supplied from the anterior chamber, glucose is vital in maintaining the high glycogen content of the corneal epithelium. Glycogen storage is quickly depleted in disease, as well as during mechanical stress such as prolonged contact lens wear.[21] Therefore, the first concern is with the possible effects of intrastromal PVA on the glycogen content of overlying epithelium. Observation of the corneal surface by biological slit lamp microscope following PVA insertion shows that the epithelium in implanted rabbits is intact, demonstrated by the exclusion of fluorescein dye (Figure 1). Histology sections show a well-differentiated, stratified epithelium 1 month following insertion, and PAS staining showed that epithelial cells contain similar glycogen content as the normal control animal. The implanted PVA-COL disk therefore did not show signs of metabolic stress in the epithelium.

Human epithelial cells were then cultured on the PVA-COL polymer by air-lift culture, which is a three-dimensional epithelium-mesenchymal coculture technique.[22–24] Confluent epithelial cells are cultured at an air–liquid interface, while feeder fibroblasts are submerged in the medium. Nutrients in this system are only supplied from the underlying substrate, because the epithelium is not submerged in the medium. Thus, the permeability of PVA-COL was shown to support a stratified epithelium *in vitro* as well. In this study, 6 out of 9 trials resulted in the stratification of human epithelium on PVA-COL following air-lift cultures, which is comparable to air-lift cultures on biological scaffolds such as the amniotic membrane.[25]

Stratified human epithelium on PVA-COL expressed the cornea-specific differentiation marker keratin 3/12, and the

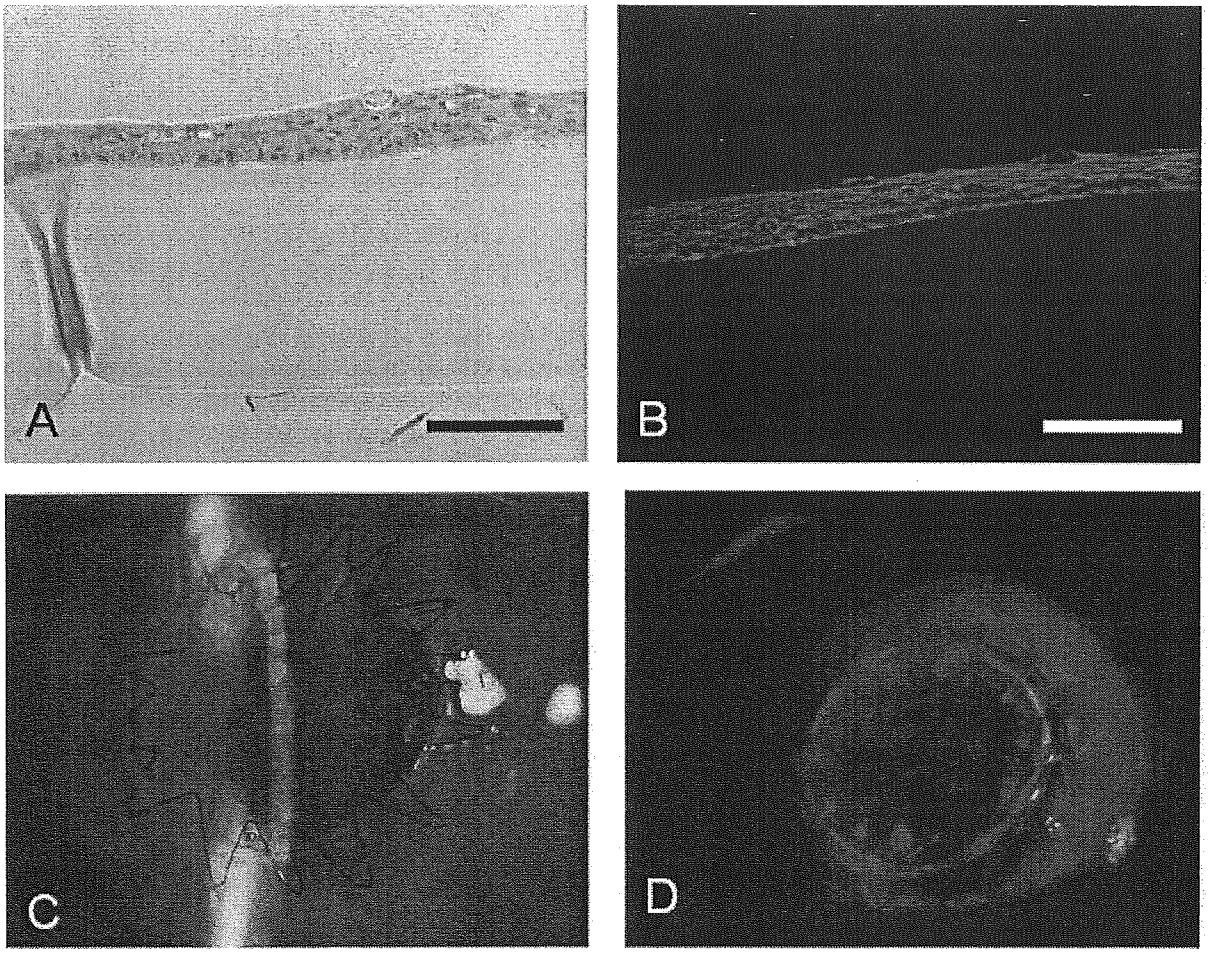

Figure 6. Rabbit corneal epithelial cells stratified on PVA-COL. (A) HE-stained section of the PVA-COL with rabbit stratified epithelium prior to transplantation. (B) Antikeratin 3/12 staining (red). Nuclei were stained with DAPI (blue). (C,D) Slit lamp photograph of PVA-COL with the epithelium immediately after the transplantation as a lamellar graft. Bar = 100 μm.

tight-junction–associated protein, occludin. Function of superficial cell tight junctions was also shown to be intact by the inhibition of horseradish peroxidase intrusion. EM showed that the superficial cells were equipped with microvilli-like structures observed in healthy corneal epithelia. These results indicate that the stratified epithelium on PVA-COL has the same histological and functional characteristics as the healthy epithelial surface. An intact barrier function is crucial in defending the cornea from microbial invasion, which in the case of artificial corneas can lead to loss of the prosthesis.

Although the superficial human epithelium of the PVA-COL composite showed all the features of a differentiated corneal epithelium, the basal cells did not express basement membrane components such as collagen type IV and collagen type VII, despite the addition of soluble laminin and the protease inhibitor aprotinin. It is possible that the period of air-lift culture was too short for these components to become apparent by immunohistology. Two strategies to improve basement membrane deposition are under investigation. One is to crosslink a thicker layer of collagen, and another is to crosslink a natural basement membrane component such as a denuded segment of amniotic membrane.

Stratified PVA-COL rabbit epithelium composites were transplanted to the rabbit cornea. The PVA-COL with stratified epithelium excluded fluorescein immediately after transplantation indicating an intact epithelial barrier. The grafts were easy to maneuver, with a texture similar to a donor cornea. However, unlike the human cornea, inflammation caused sutures to become loose, and detachment of the epithelium was observed after a few days. These results suggest that although PVA-COL can sustain the epithelium *in vitro*, modification of surgical procedure will be required to sustain the epithelialized scaffold *in vivo*. A modified hybrid PVA polymer is also being developed in order to allow the formation of the basement membrane, which was not observed with PVA-COL. A healthy basement membrane complex may further improve the durability of the epithelium following transplantation.

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The Implications of the Upregulation of ICAM-1/VCAM-1 Expression of Corneal Fibroblasts on the Pathogenesis of Allergic Keratopathy

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Objective. The present study investigated the expression of ICAM-1 and VCAM-1 on fibroblasts with interleukin (IL)-4 and/or tumor necrosis factor (TNF)-α stimulation and assessed the effect of eosinophil adhesion on fibroblast viability.

Methods. Primary cultured human corneal fibroblasts were incubated with IL-4, TNF-α, or their combination for 24 hours. Expression of ICAM-1 and VCAM-1 was examined by real-time quantitative PCR and flow cytometric analysis. Purified eosinophils were cocultured with activated fibroblasts, and the number of eosinophils adhered to fibroblasts and the number of damaged fibroblasts were counted using microscopy. In a separate trial, conjunctival and corneal impression cytology was performed on patients with atopic keratoconjunctivitis and corneal ulcers (eight eyes) to assess the status of the ocular surface epithelium and the presence of inflammatory cell infiltrates.

Results. Real-time quantitative PCR and flow cytometric analysis revealed that both mRNA and protein of VCAM-1 and ICAM-1 were upregulated by IL-4 and TNF-α. IL-5-primed eosinophils adhered to the corneal fibroblasts treated with IL-4 and TNF-α, and the fibroblasts were damaged by eosinophil adherence. Anti-ICAM-1 antibody and anti-VCAM-1 antibody inhibited the eosinophil adherence to fibroblasts and the fibroblast damage. Impression cytology revealed extensive infiltration of neutrophil and eosinophils among isolated ocular surface epithelial cells with advanced squamous metaplasia.

Conclusions. Corneal fibroblasts expressed ICAM-1 and VCAM-1 when activated with IL-4 and TNF-α. Eosinophils can adhere to the activated fibroblasts and can induce subsequent fibroblast damage through these adhesion molecules. Eosinophil adhesion to fibroblasts may possibly contribute to the pathogenesis of severe persistent allergic corneal ulcers. (*Invest Ophthalmol Vis Sci.* 2005;46:4512–4518) DOI:10.1167/iovs.04-1494

Eosinophils are thought to exacerbate the late-phase inflammatory response in immediate-type allergic reactions by releasing leukotrienes and highly cytotoxic proteins, such as major basic protein (MBP) and eosinophilic cationic protein (ECP).[1] These proteins may cause a variety of corneal disorders, including superficial punctate keratopathy and corneal ulcer in vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC).[2–5] The presence of eosinophils and the deposition of ECP have already been observed in conjunctival tissues and in tears of patients with AKC and VKC.[6–10] MBP deposits were also observed in allergic corneal ulcers.[11] Purified MBP and ECP reduced corneal epithelial cell viability and caused morphologic changes in vitro.[12] These findings strongly suggest that eosinophils play an important role in the pathogenesis of allergic corneal ulcer. However, the precise mechanisms by which eosinophils damage the corneal tissue remain unclear.

Allergic reactions in the conjunctiva induce the release from inflammatory cells of various types of cytokines, including proinflammatory cytokines and helper type 2 T-cell (Th2) cytokines. Levels of interleukin (IL)-4 and tumor necrosis factor (TNF)-α in the tears of patients with allergies were found to be significantly higher than in healthy subjects.[13,14] These cytokines are known to modulate various functions of fibroblasts, such as eotaxin production and adhesion molecule expression.[15,16] Eotaxin is known to induce further recruitment of eosinophils.[17] On the other hand, adhesion molecules are thought to play an important role in the binding of eosinophils, through which eosinophils are believed to be stimulated further to release inflammatory mediators.[18,19]

Recently, it has been found that CD11/18-dependent adhesion is a critical step in human eosinophil degranulation.[20,21] Eosinophils express all four members of the CD18 (β2) leukocyte integrin family, CD11a to -d, which allow them to bind to their ligands, ICAM-1 to -3.[22–27] Eosinophils also express CD49d/CD29, which bind to the ligand VCAM-1.[28] In human lung fibroblasts, IL-4- and TNF-α-dependent expression of ICAM-1 and VCAM-1 and the influence of eosinophil-fibroblast adhesion on eosinophil degranulation have been reported.[29] However, it is still unclear how the eosinophil-fibroblast interactions influence allergic corneal inflammation, especially the course of corneal ulcer formation. We believed that actual adhesion of eosinophils to corneal fibroblasts through ICAM-1 and/or VCAM-1 might induce subsequent activation and might contribute to the evolution of persistent allergic corneal ulcers. Therefore, we initially looked into the changes of expression of ICAM-1 and VCAM-1 on stimulating corneal fibroblast cultures by IL-4 and TNF-α by employing flow cytometry and real-time PCR. We also cocultured corneal fibroblasts with eosinophils to assess the adhesion between the two cell types. We then investigated the timewise cell damage on corneal fibroblasts after eosinophil binding, as well as the effects of anti-ICAM-1 and anti-VCAM-1 applications on eosinophil adhesion and eosinophil-induced damage to the corneal fibroblasts. In addition, we performed conjunctival and corneal impression cytology on patients with AKC and corneal ulcers to evaluate the status of the ocular surface epithelium and the presence of eosinophils in the inflammatory response, if any.

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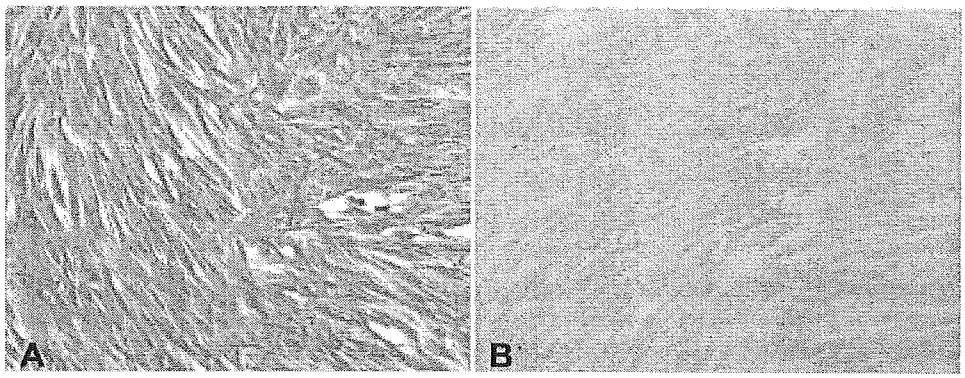

FIGURE 1. Immunohistochemistry staining of corneal fibroblasts by antivimentin antibodies. (A) Image shows diffuse positive staining with antivimentin antibodies (rabbit polyclonal, 1:2000 dilution; Lab Vision Corporation, Fremont, CA) and a lack of impurities from the presence of any other cell types in the slide chamber (magnification 200×). (B) Image shows the absence of staining in the negative control specimen (magnification 200×).

MATERIALS AND METHODS

Primary Cell Cultures

Three human corneas were obtained from the American Eye Bank Association. Briefly, after the center of each cornea was punched out for transplantation, the remaining rim of tissue was used to prepare corneal fibroblasts. The human tissue was used in strict accordance with the tenets of the Declaration of Helsinki. Corneal tissue was cut into pieces and then placed on collagen-coated 35-mm culture dishes (Iwaki, Tokyo, Japan). Corneal fibroblasts were isolated from corneal tissue explants and cultured with fibroblast culture medium composed of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM-F12; Gibco-BRL, Grand Island, NY), supplemented with 10% fetal calf serum (FCS; Gibco), 100 IU/mL penicillin, and 100 μg/mL streptomycin (all supplied by Invitrogen-Gibco Life Technologies, Paisley, UK) at 37°C with 5% (vol/vol) CO_2 in air. Cultures of passages 3 and 6 were used in the present study. The purity of the cell cultures was assessed on the basis of both the distinctive morphology of corneal fibroblasts and their reactivity with antibodies to vimentin and isotype control in immunohistochemistry analysis. No contamination by corneal epithelial cells was detected (Fig. 1).

Isolation of Human Eosinophils

Human granulocytes were isolated from heparin-anticoagulated venous blood of atopic volunteers. The granulocytes underwent Percoll density gradient centrifugation (490*g*) at room temperature, and CD16-positive cells were removed using immunomagnetic beads (Miltenyi Biotec GmbH, BergischGladbach, Germany) as previously described.[30] Eosinophil purity of cytocentrifuge preparations was determined by staining (Diff-Quick; American Scientific Products, McGraw Park, IL) and was confirmed to be greater than 98%.

Quantitative Real-Time PCR

We confirmed whether ICAM-1 and VCAM-1 mRNA expressions were correlated with the concentrations of IL-4 and TNF-α by performing quantitative measurements of ICAM-1 and VCAM-1 mRNA with real-time PCR. The primary human corneal fibroblasts from three different donors were cultured for 24 hours with various cytokine concentrations, after which total RNA was extracted. RNA was extracted from corneal fibroblasts cultured in the presence or absence of 0.3 to 30 ng/mL IL-4 and TNF-α for 24 hours. A commercially available sequence detection system (ABI PRISM 7700; Applied Biosystems, Warrington, UK) and gene expression assay mixes (TaqMan Universal PCR Master Mix and Assay-on-Demand Gene Expression Assay Mix; Applied Biosystems) were used for real-time quantitative PCR to measure for ICAM-1, VCAM-1, and GAPDH. The thermal profile consisted of 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds and 60°C for 1 minute. Results were analyzed by the comparative cycle threshold method.[31,32]

Flow Cytometry Analysis

To examine the ICAM-1 and VCAM-1 protein expression on the cell surface of cultured fibroblasts, flow cytometric analysis was performed 24 hours after cytokine stimulation. IL-4– and/or TNF-α–stimulated corneal fibroblasts were gently removed from the six well-culture dishes with cell dissociation buffer (Gibco-BRL), washed, and diluted in PBS containing 1% BSA and 0.1% NaN_3. Cells were stained by monoclonal antibodies against mouse IgG_1 (Sigma, St. Louis, MO), ICAM-1 (84H10; Immunotech, Marseille, France) or VCAM-1 (1G11; Immunotech), respectively, and analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lane, NJ) and analysis software (CellQuest; Becton Dickinson, Mountain View, CA).

Eosinophil-Fibroblast Adhesion Assay

To determine the functional significance of ICAM-1 and VCAM-1 expression on cultured fibroblasts, in vitro eosinophil adhesion experiments were performed. Corneal fibroblasts from three different donors were cultured in 96-well culture plates (Becton-Dickinson Labware, Lincoln Park, NJ) for 48 hours After starvation for 24 hours, the cells were stimulated with IL-4 (1–100 ng/mL) and/or TNF-α (1–100 ng/mL)

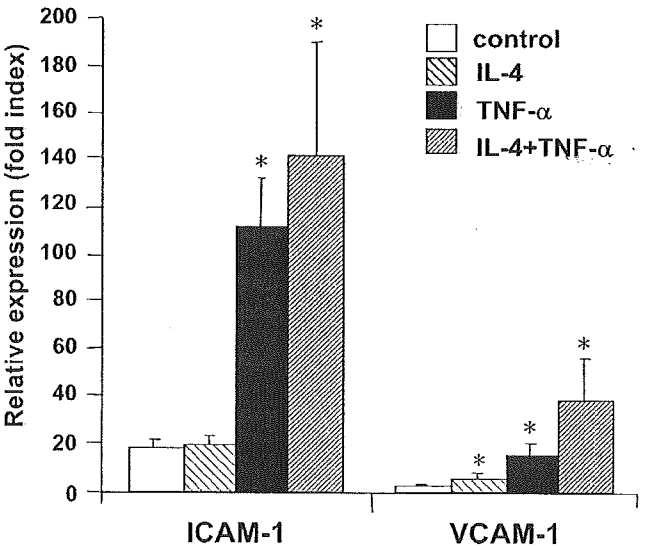

FIGURE 2. Relative expression of mRNA encoding ICAM-1 and VCAM-1 on corneal fibroblasts compared to controls. Corneal fibroblasts were incubated with IL-4 (0.3–30 ng/mL), TNF-α (0.3–30 ng/mL), or their combination (0.3–30 ng/mL) for 24 hours Total RNA was isolated and real-time quantitative PCR for ICAM-1, VCAM-1, and GAPDH was performed. Data were analyzed by the comparative cycle threshold method and expressed as the fold index relative to the nonstimulated control (three trials). Values are means ± SD; $^*P < 0.05$, Student's *t*-test.

for 24 hours. Purified eosinophils from three different donors were preincubated with 1 ng/mL IL-5 for 15 minutes, then cocultured with the cultured fibroblasts (1×10^5 cells per well) for 3 hours The wells were washed gently with PBS to remove nonadhered eosinophils, and the eosinophils adhered to the fibroblasts were counted using the light microscope field. To determine the effect of two adhesion molecules, ICAM-1 and VCAM-1, on eosinophil-fibroblast interactions, an inhibition assay was performed using anti–ICAM-1 monoclonal antibody (mAb) and anti–VCAM-1 mAb. After stimulation of fibroblasts with IL-4 and TNF-α for 24 hours, 50 μL anti-mouse IgG_1 (Sigma), anti–ICAM-1 mAb, and anti–VCAM-1 mAb (Immunotech) were added and reacted for 30 minutes at 37°C before coculturing.

Morphologic Study of Fibroblasts Cocultured with Eosinophils

By a similar method as the adhesion assays, fibroblasts were cultured in 96-well plates and stimulated with IL-4 and/or TNF-α for 24 hours. Preactivated eosinophils were added to the cultured fibroblasts, and they were cocultured for 72 hours. After coculturing, fibroblasts were gently washed twice with PBS, then 20 μL of 0.5% trypan blue solution was added, and the cells were stained for 1 minute. After removing the solution with a pipet, nonstained cells, regarded as intact, were immediately counted under the microscope.

TUNEL Assay for Detection of Apoptosis in Fibroblasts

Apoptosis in the cocultures of fibroblasts with eosinophils was detected employing the TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuridine triphosphate [dUTP] nick-end labeling) assay, using a commerically available kit (In Situ Cell Death Detection Kit, Peroxidase; Boehringer Mannheim, Mannheim, Germany) per manufacturer's instructions.

Repeatability of the Individual Experiments

Gene chip analysis, real-time PCR and flow cytometry experiments, adhesion assays, and morphologic studies were repeated three times in this study.

Conjunctival and Corneal Impression Cytology

Conjunctival and corneal impression cytology was performed on eight patients with AKC and corneal ulcers (8 eyes; 7 males, 1 female; mean age 24 years, range 9–34 years). The impression cytology specimens were obtained after administration of topical anesthesia with 0.4% oxybuprocaine. Strips of cellulose acetate filter paper (HAWP 304; Millipore, Bedford, MA) that were soaked in distilled water for a few hours and dried at room temperature were applied on upper palpebral conjunctiva and corneal ulcers, pressed gently by a glass rod, and then removed. The specimens were then fixed with formaldehyde. The specimens were stained with periodic acid schiff, dehydrated in ascending grades of ethanol and then with xylol, and finally coverslipped. The status of epithelial cells was determined by taking photographs using a light microscope at a magnification of 400×. The same researcher, who was masked to the identity of the specimen donors, evaluated the specimens for goblet cell counts, epithelial squamous metaplasia grades, and presence of inflammatory cell infiltrates.

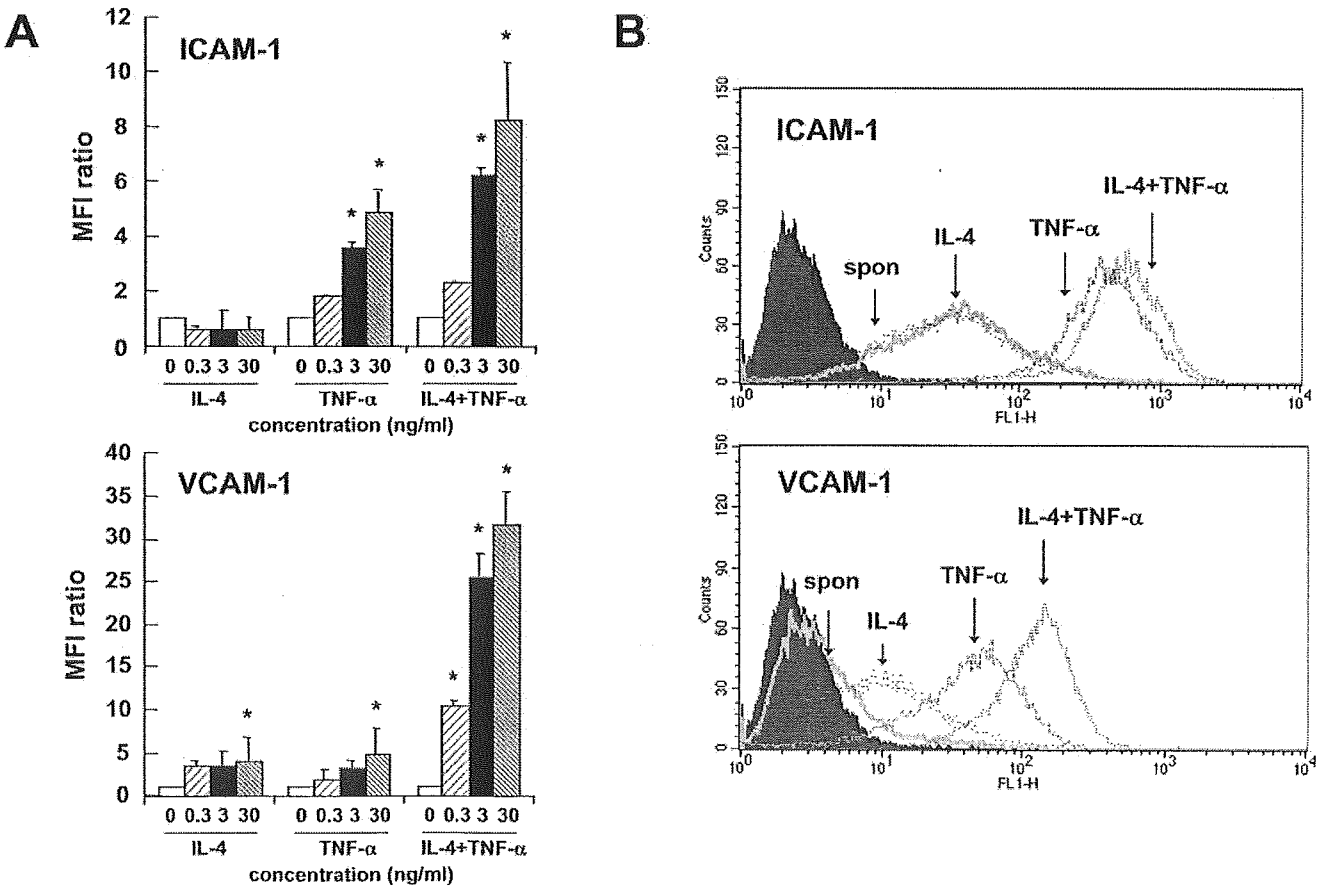

FIGURE 3. Flow cytometry analysis of the expression of ICAM-1 and VCAM-1 on corneal fibroblasts. Corneal fibroblasts were incubated with IL-4 (0.3–30 ng/mL), TNF-α (0.3–30 ng/mL), or their combination (0.3–30 ng/mL) at 37°C for 24 hours. Cells were washed and stained with anti–ICAM-1 mAb and anti–VCAM-1 mAb and analyzed by flow cytometry. IL-4 and TNF-α induced the expression of ICAM-1 and VCAM-1 on corneal fibroblasts. (**A**) Mean fluorescence intensity (MFI) of ICAM-1 and VCAM-1 in flow cytometry ($n = 3$); $^*P < 0.05$. (**B**) Representative individual fluorescence intensity profile of ICAM-1 and VCAM-1 in flow cytometry.

Statistical Analysis

All data are presented as means ± SEM. Depending on the distribution of the individual data, the significance of differences between groups was determined by either Student's *t*-test or a Mann–Whitney rank sum test. Repeated-measures ANOVA was used where more than one comparison was made. Values of $P < 0.05$ were considered significant.

Results

Real-Time PCR Analysis of ICAM-1 and VCAM-1 Expression after IL-4 and TNF-α Stimulation of Fibroblasts

A significant increase in both ICAM-1 and VCAM-1 mRNA expression over basal levels was observed with TNF-α (0.3–30 ng/mL) stimulation (4.8- and 4.6-fold, compared to control, at 30 ng/mL) as shown in Figure 2. Although IL-4 (0.3–30 ng/mL) alone did not enhance ICAM-1 mRNA expression, a combination of TNF-α and IL-4 showed a slight additive effect on ICAM-1 mRNA expression. In contrast, VCAM-1 mRNA expression was increased by IL-4 alone to the same extent as with TNF-α stimulation (4.0-fold, at 30 ng/mL). A combination of 30 ng/mL TNF-α and IL-4 resulted in the greatest augmentation of VCAM-1 mRNA expression (32-fold).

Flow Cytometry Analysis of ICAM-1 and VCAM-1 Expression on Corneal Fibroblasts

As shown in Figure 3, ICAM-1 was constitutively expressed on corneal fibroblasts, and significantly enhanced by TNF-α stimulation alone ($P < 0.05$) but not by IL-4 stimulation. Stimulation with the combination of IL-4 and TNF-α showed an additive effect on ICAM-1 expression. On the other hand, VCAM-1 was scarcely expressed on resting corneal fibroblasts, but was induced by IL-4 or TNF-α stimulation. A combination of IL-4 and TNF-α showed an additive effect on VCAM-1 expression.

Morphologic Analysis of Eosinophil Adhesion to Corneal Fibroblasts

When unstimulated fibroblasts were incubated with activated eosinophils (1×10^5 cells per well), slight spontaneous eosinophil adhesion to fibroblasts was noted at 20.3 ± 4.9 cells/high power field (hpf) (Fig. 4A). After preincubation of fibroblasts with IL-4 (1–100 ng/mL) or TNF-α (1–100 ng/mL) for 24 hours, the number of adhered eosinophils increased in a dose-dependent manner ($P < 0.001$). A combination of IL-4 and TNF-α showed an additive effect on eosinophil adhesion. After stimulation with both 100 ng/mL IL-4 and 100 ng/mL TNF-α, eosinophil adhesion was most enhanced at 99.0 ± 15.6 cells/hpf (a fivefold increase, compared with nonstimulated controls).

Morphologic Analysis of the Viability of Corneal Fibroblasts Cocultured with Eosinophils

In eosinophil adhesion assays, we observed slight morphologic changes in fibroblasts cocultured with eosinophils after 3 hours of incubation. Therefore, we continued the coculture study and observed the eosinophil-dependent morphologic changes in the fibroblasts. At 72 hours, corneal fibroblasts were partially damaged, and a number of cells were detached from the culture dish. After cocultured fibroblasts were gently washed with PBS, a trypan blue exclusion test was performed. The number of intact fibroblasts incubated without eosinophils was 44.0 ± 3.4 cells/hpf, and the viability was consistently 100%. It was also confirmed that IL-4 and TNF-α scarcely affected the viability of fibroblasts in our system (data not shown). Preincubation of fibroblasts with IL-4 or TNF-α increased the number of damaged cells in a concentration-dependent manner when cocultured with eosinophils (Fig. 4B). Preincubation with both 100 ng/mL IL-4 and 100 ng/mL TNF-α showed an additive effect on cell damage (53.7%).

All fibroblasts in IL-4– and TNF-α–stimulated cocultures with eosinophils were TUNEL negative and did not reveal specific features of apoptosis, such as shrinkage of cells or nuclear changes.

Morphologic Changes of Eosinophil Adhesion to Corneal Fibroblasts and Eosinophil-Induced Fibroblast Damage with Anti–ICAM-1 or Anti–VCAM-1 Treatment

The addition of anti–ICAM-1 mAb or anti–VCAM-1 mAb (10 μg/mL) significantly inhibited the adhesion of eosinophils to

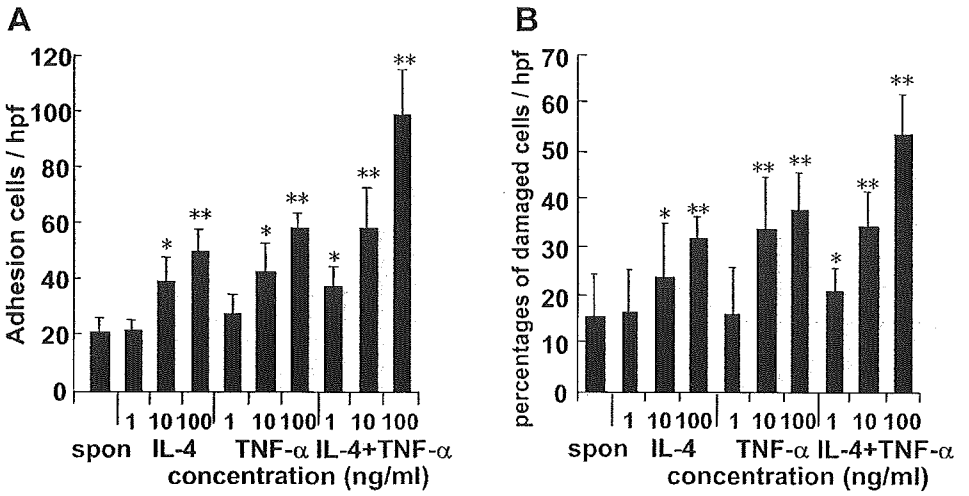

FIGURE 4. Morphologic analysis of eosinophil adhesion and fibroblast damage. Corneal fibroblasts were preincubated for 24 hours with IL-4 and/or TNF-α. Eosinophils primed with IL-5 were then added. (A) Graph shows the number of adhered eosinophils counted by light microscopic observation; eosinophil adhesion to corneal fibroblasts is significantly increased in stimulated cultures compared to control cultures ($n = 3$); $^*P < 0.05$, $^{**}P < 0.005$. (B) Eosinophils were incubated for 72 hours. Graph shows a significant increase of corneal fibroblast damage compared to control cultures. Percentages of damaged cells were calculated by subtraction of trypan blue–negative cells (100% = viability of control fibroblasts without addition of eosinophils and cytokines; $n = 3$); $^*P < 0.05$, $^{**}P < 0.005$. spon, spontaneous.

activated fibroblasts (Fig. 5A). Preincubation with anti-ICAM-1 or anti-VCAM-1 mAb significantly inhibited the eosinophil adhesion by approximately 80%, compared to the level observed with mouse IgG_1 application.

Eosinophil-induced cell damage was also assayed after 72 hours of coculturing. Treatment with anti-ICAM-1 mAb and anti-VCAM-1 mAb significantly reduced the fibroblast damage (57% and 43%, respectively) to a similar extent as with eosinophil adhesion (Fig. 5B). An additive reduction of fibroblast damage was observed when fibroblasts were treated with both anti-ICAM-1 and anti-VCAM-1 mAbs.

Conjunctival and Corneal Impression Cytology

To provide further evidence for our in vitro findings, conjunctival and corneal impression cytology was carried out on eight patients with AKC and sterile shield corneal ulceration (eight eyes). Conjunctival imprints from all eyes contained sheets of conjunctival epithelial cells with advanced squamous metaplasia, inflammatory cell infiltrates, predominant neutrophils and eosinophils, variable amounts of goblet cells, and mucin pickup. Figure 6A shows an abundance of inflammatory cell infiltrates, consisting mainly of neutrophils and eosinophils, adjacent to conjunctival epithelial cells with advanced squamous metaplasia (Dogru M, et al. *IOVS* 2002;46:ARVO E-Abstract 939). The red arrows indicate eosinophils, the black arrow points to a conjunctival epithelial sheet with Nelson's grade 2 squamous metaplasia and decreased cellular cohesion, and the black stars indicate a total loss of cellular cohesion between the focus of inflammation and the surrounding epithelial cells. The orange arrow indicates a loss of cellular cohesion between the conjunctival epithelial cells lying under the inflammatory infiltrates.

Imprints from eyes of patients with corneal ulcers revealed extensive infiltration by both neutrophils and eosinophils among isolated corneal epithelial cells with advanced squamous metaplasia. Figure 6B, a representative corneal impression cytology imprint obtained from the ulcer edge in the same patient as Figure 6A, shows extensive infiltration by eosinophils (red arrows) and neutrophils and isolated corneal epithelial cells with Nelson's grade 3 squamous metaplasia.

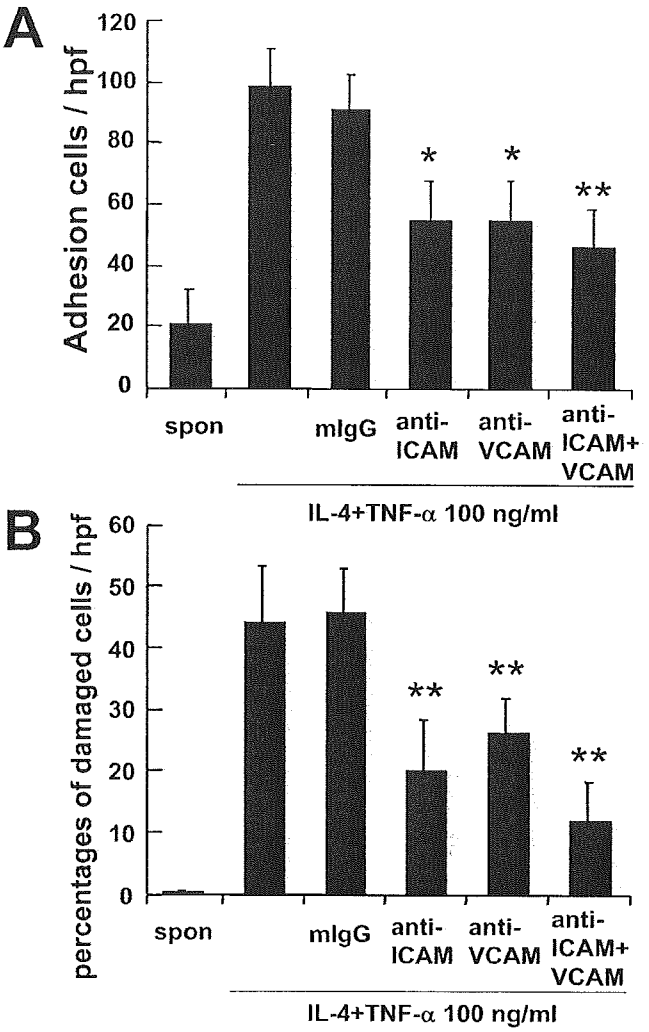

FIGURE 5. The effects of anti-ICAM-1 and anti-VCAM-1 mAb on cell adhesion and fibroblast damage. Cultured fibroblasts were treated with IL-4 and TNF-α (100 ng/mL each) and mouse IgG1 (mIgG; control), anti-ICAM-1, and anti-VCAM-1 antibody (10 mg/mL each) for 30 minutes. (A) Primed eosinophils were cocultured for 3 hours, and adhered eosinophils were counted ($n = 3$); $^*P < 0.05$, $^{**}P < 0.005$. (B) Primed eosinophils were cocultured for 72 hrs, and the viability of keratocytes was evaluated by counting trypan blue-negative cells. Percentage of damaged cells was calculated by subtraction of trypan blue-negative cells ($n = 3$); $^{**}P < 0.005$. spon, spontaneous.

DISCUSSION

Eosinophils have long been blamed for the morbidity of corneal complications in patients with allergies. Indeed, eosinophils have been shown to accumulate on the ocular surface in patients with allergic conjunctivitis and to exert cytotoxic effects through degranulation. We have previously reported higher concentrations of eotaxins in the tears of patients with severe ocular allergies and observed that tears of such patients had chemotactic activity for eosinophils in vitro.[6] Although the current literature suggests some evidence in relation to eosinophil-induced ocular damage in ocular surface allergies, the precise mechanisms of the formation of severe corneal damage, especially corneal ulceration, are still not clear.

Therefore, we performed quantitative real-time PCR and flow cytometric analysis to confirm the expression of ICAM-1 and VCAM-1 related to eosinophils at the mRNA and protein levels. We also looked into the in vitro morphologic alterations of corneal fibroblasts cocultured with eosinophils by assessing eosinophil adhesion to corneal fibroblasts, fibroblast viability, and the effects of anti-ICAM-1 and anti-VCAM-1 treatment on fibroblast morphology.

It is well known that keratocyte apoptosis is one the first events to follow corneal epithelial injury. Virtually any source of corneal epithelial injury, such as a mechanical scrape, LASIK surgery, or an infectious process, causes the release of cytokines from the epithelium, resulting in the activation and transformation of keratocytes into a repair phenotype of fibroblasts that release enzymes such as collagenases, matrix metalloproteinases involved in stromal modeling.[33] We thus chose to use fibroblast instead of keratocyte cultures in this study to investigate active fibroblast-related events that may be important in allergic wound-healing response. To support the findings from the in vitro experiments of this study, we performed conjunctival and corneal impression cytology on AKC patients with corneal ulcers as well.

We initially confirmed the expression of two adhesion molecules, ICAM-1 and VCAM-1, by real-time PCR and flow cytometry. Real-time PCR provided evidence that ICAM-1 and VCAM-1 mRNA expression were upregulated with IL-4 and TNF-α stimulation. Flow cytometry analysis revealed further evidence of increased corneal fibroblast expression of ICAM-1 and VCAM-1 on stimulation by IL-4 and TNF-α. We thought that increased expression of ICAM-1 and VCAM-1 on corneal fibroblasts on stimulation with IL-4 and TNF-α, two cytokines

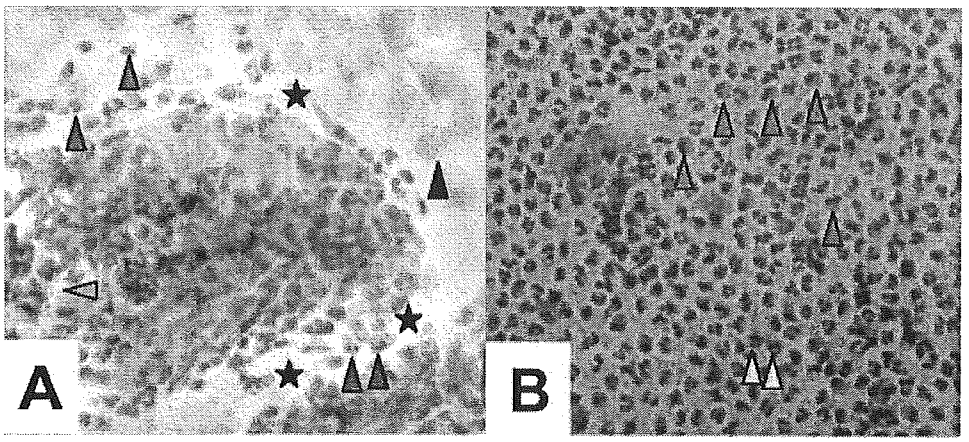

FIGURE 6. (A) Conjunctival impression cytology specimen from a patient with AKC and corneal ulceration, showing the abundance of inflammatory cell infiltrates, consisting mainly of neutrophils and eosinophils (*red arrows*), adjacent to conjunctival epithelial cells with advanced Nelson's grade 2 squamous metaplasia and decreased cellular cohesion (*black arrow*), as well as the total loss of cellular cohesion between the focus of inflammation and the surrounding epithelial cells (*black stars*) and the loss of cellular cohesion between the conjunctival epithelial cells lying under the inflammatory infiltrates (*orange arrow*) (magnification, ×100). (**B**) Representative corneal impression cytology imprint obtained from the ulcer edge in the same patient, showing extensive eosinophilic infiltration (*red arrows*) and neutrophils (*yellow arrows*) and isolated corneal epithelial cells with Nelson's grade 3 squamous metaplasia (Wiegert's iron hematoxylin staining; magnification, ×100) (Dogru M, et al. *IOVS* 2002;46:ARVO E-Abstract 939).

known to be present in high amounts in allergic ocular surfaces, prepared a background which would ease the adhesion of eosinophils to corneal fibroblasts.

Indeed, eosinophils are known to bind ICAM-1 via all four members of the CD18 integrin family, CD11a to -d, which are expressed on the eosinophil cell sulface.[22–27] Eosinophils also express a number of $\beta 1$ integrins, of which $\alpha 4\beta 1$, the ligand of VCAM-1, is best characterized.[28] Observations from previous studies suggest that ICAM-1 and VCAM-1 are the potent molecules involved in eosinophil-fibroblast adhesion. The present study is the first to describe the morphologic alterations of corneal fibroblasts exposed to IL-4 and TNF-α, and bound by eosinophils.

Our morphologic observations of the cocultures showed that both IL-4 and TNF-α increased eosinophil adhesion to corneal fibroblasts solitarily and in combination. Likewise, applications of both cytokines were associated with significant increases in corneal fibroblast cell damage in the cultures. Our TUNEL assay findings suggested that fibroblast cellular damage was due to eosinophil-induced necrosis rather than apoptosis. Interestingly, blocking experiments performed with anti-ICAM-1 and anti-VCAM-1 applications revealed partial reversal of cellular damage. The reversal effect was enhanced when anti-ICAM-1 and anti-VCAM-1 were applied concomitantly.

Our results suggest that eosinophil adhesion to corneal fibroblasts via ICAM-1 and VCAM-1 may lead to degranulation of eosinophils. Indeed, eosinophils contain cytotoxic proteins such as ECP and MBP, which are released on activation and have been reported to cause damage to corneal cellular structures.[12] However, it should be noted that eosinophil adhesion was not completely inhibited with the anti-ICAM-1 and anti-VCAM-1 monoclonal antibodies in this study, indicating that other molecules may be associated with corneal fibroblasts. Recent evidence indicates that cell adhesion through CD11b/CD18 (Mac-1) is a crucial step for the activation, signaling, and effector function of eosinophils.[20,21] On the other hand, it has been reported that VLA-4 (CD49d)-mediated adhesion augments stimulated eosinophil degranulation. Therefore, ICAM-1/$\beta 2$ and VCAM-1/VLA-4 interactions seem to be involved in the mechanisms of eosinophil cytotoxicity.

Conjunctival impression cytology findings in all subjects revealed inflammatory cell infiltrates, consisting mainly of neutrophils and eosinophils, adjacent to conjunctival epithelial cells with advanced squamous metaplasia, providing clinical evidence on the adverse effects of the inflammatory process on the epithelial cells and backing up our findings from the in vitro experiments. Interestingly, imprints obtained from corneal ulcers showed extensive eosinophilic and neutrophilic infiltration among isolated corneal epithelial cells with advanced squamous metaplasia, suggesting adverse effects of the infiltrates on cellular cohesion and integrity.

In conclusion, we found that corneal fibroblasts express ICAM-1 and VCAM-1 when activated with IL-4 and TNF-α, and the expression is highly selective among all adhesion-related molecules. Eosinophils can adhere to the activated fibroblasts and can induce subsequent fibroblast damage through the adhesion molecules. Eosinophil adhesion to fibroblasts may contribute to the pathogenesis of severe persistent allergic corneal ulcers.

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Clinical Evaluation of the Accuracy of Intraocular Pressure Measurement by Tono-Pen XL in Eyes With Amniotic Membrane Patching

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PURPOSE: To evaluate the influence of amniotic membrane on intraocular pressure (IOP) measurement with the Tono-Pen.

DESIGN: Observational case series.

METHOD: Eight patients with partial limbal dysfunction who underwent penetrating keratoplasty were recruited. Amniotic membranes were patched just after keratoplasty. One week postoperatively, amniotic membranes were removed, and accuracy of IOP measurement with the Tono-Pen was evaluated with comparisons before and after the removal.

RESULTS: No epithelial defects were detected after the removal of amniotic membrane. Mean IOPs with and without amniotic membranes were 16.6 ± 3.0 mm Hg (mean ± SD) and 16.0 ± 3.7 mm Hg, respectively. The difference was not statistically significant.

CONCLUSIONS: Assessment of IOP with the Tono-Pen was observed to be accurate even when used over an amniotic membranes patch in cases who underwent penetrating keratoplasty. (Am J Ophthalmol 2005;139: 570–571. © 2005 by Elsevier Inc. All rights reserved.)

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AMNIOTIC MEMBRANE (AM) HAS BEEN REPORTED TO promote corneal epithelialization and has found widespread use in the treatment of persistent epithelial defects.[1,2] Once an AM patch has been applied, accurate assessment of intraocular pressure (IOP) with the Goldmann applanation tonometer becomes difficult. One of the few options for the measurement of IOP in such cases is the Tono-Pen XL (Medtronic Solan, Florida, USA). It has been reported that a single layer of an AM patch did not affect the accuracy of Tono-Pen-aided IOP measurements in rabbit eyes.[3] We evaluated the influence of AM patching on the accuracy of IOP measurements with this instrument in human eyes for the first time in a clinical setting.

Eight patients with partial limbal deficiency undergoing penetrating keratoplasty (PKP) were enrolled in this study, as shown in Table 1. The patients were informed of the risks and benefits of AM patching and gave their written informed consent. The study adhered to the guidelines of the Declaration of Helsinki. AMs were sutured with the epithelial side facing upward to cover the whole cornea. One week postoperatively, the AM was removed. IOP measurements were made five times before and after removal. A paired *t* test was performed for statistical analysis. Measurements of IOP by Goldmann applanation tonometer was also performed after removal.

On removal of the AM, complete epithelialization of the graft was observed in all cases. Mean IOPs with and without AM were 16.6 ± 3.0 mm Hg (mean ± SD) and 16.0 ± 3.7 mm Hg, respectively. There were no significant differences between the IOP measurements with

TABLE 1. Sex, Age, Etiologic Distribution and Intraocular Pressure Measurement

				Mean IOP ± SD (mmHg)		
				Tono-Pen		
Case	Sex	Age	Original Disease	with AM	without AM	GAT without AM
1	M	76	Bullous keratopathy	18.7 ± 3.5	18.0 ± 0.0	N.A.
2	M	65	Corneal opacity	19.7 ± 1.2	20.0 ± 1.7	N.A.
3	M	37	Bullous keratopathy	14.2 ± 0.8	14.8 ± 1.8	15.0 ± 0.0
4	M	78	Bullous keratopathy	12.4 ± 3.5	12.0 ± 0.7	13.0 ± 0.0
5	M	42	Bullous keratopathy	20.2 ± 2.1	21.2 ± 1.3	18.0 ± 0.0
6	F	68	Corneal opacity	14.0 ± 1.4	12.8 ± 0.8	13.0 ± 0.0
7	F	69	Bullous keratopathy	15.6 ± 0.9	11.8 ± 1.1	13.6 ± 0.6
8	F	54	Bullous keratopathy	18.4 ± 0.9	17.3 ± 1.5	N.A.
		Average		16.6 ± 3.0	16.0 ± 3.7	14.5 ± 2.1

and without AM. In clinical practice, we believe a tonometry error of 3 mm Hg would be significant. Our series of eight patients provided a 99% power to detect such a difference. In case 7, the IOP values with AM was more than 3 mm Hg higher compared with without AM. In this case, the sutures loosened postoperatively, and the AM was not sufficiently taut. This suggests that insufficient tension of the AM patch may affect IOP measurement using the Tono-Pen.

PKP is often complicated by glaucoma, and some patients experience transient postoperative IOP elevation. Thus, accurate IOP measurement is of utmost importance. Rao and associates[4] reported that the accuracy of the Tono-Pen was comparable to Goldmann applanation tonometry in postkeratoplasty eyes. Mok and associates[5] reported that the thickness of cornea does not affect Tono-Pen readings.

Persistent corneal epithelial defects after PKP should be avoided to minimize postoperative infection. Inflammation caused by persistent epithelial defects may lead to graft rejection, opacification, and eventual failure. AM patching has become an important treatment modality in patients with persistent epithelial defects caused by limbal deficiency. Nevertheless, the alignment of two semicircles of Goldmann applanation tonometer is unobtainable over the AM, and thus this method of IOP measurement is unsuitable for such patients.

In this study, the Tono-Pen was found to be accurate even when used over an AM patch in cases that underwent PKP. We believe that the Tono-Pen is probably the most viable and accurate option for measuring IOP in cases with AM patching.

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Voluntary Alteration of Full-field Electroretinogram

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PURPOSE: To evaluate whether a normal subject can deliberately produce abnormal electroretinogram (ERG) results that simulate disorders of the retina.
DESIGN: Prospective study.
METHODS: Five normal subjects were evaluated twice with full-field ERGs. The subjects were randomly assigned to be compliant or noncompliant (i.e., to deliberately attempt to alter the test results) at each visit. Results from compliant eyes were compared with those of noncompliant eyes.
RESULTS: While the amplitudes of all parameters were decreased in the noncompliant group, the difference was not statistically significant, and the results were usually within the normal limits of our laboratory. No subject was able to alter the results to mimic a retinal disease or to reproduce a specific pattern of changes.
CONCLUSIONS: Although ERG results can be altered by noncompliant subjects, the results are usually only "borderline abnormal" and do not correspond to any pattern of retinal disease. These results confirm that abnormal full-field ERGs indicate true retinal disease. (Am J Ophthalmol 2005;139:571–572. © 2005 by Elsevier Inc. All rights reserved.)

VISUAL EVOKED POTENTIAL (VEP) AND FULL-FIELD electroretinogram (ERG) are often obtained in patients with unexplained visual loss. It is well documented that the VEP can be altered voluntarily.[1] Although a recent study showed that multifocal ERG can be voluntarily suppressed,[2] many assume that abnormal full-field ERG results are always indicative of true retinal disease.[3] However, test-retest variability of full-field ERG has been demonstrated in normal subjects.[4] The objective of this study was to evaluate whether a normal subject can intentionally produce

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SCIENTIFIC REPORT

Transplantation of corneal endothelium with Descemet's membrane using a hyroxyethyl methacrylate polymer as a carrier

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Aims: To evaluate the histology and function of Descemet's membrane transplanted with intact endothelium.

Methods: Japanese white rabbits and human eyebank eyes were used as donors and recipients of Descemet's membrane transplantation. Donor endothelium was hydrodissected by injecting indocyanine green from a limbal incision, and then processed as a corneal scleral button. A 6 mm diameter donor sheet was trephined, and folded in half using a 6 mm diameter polymer as a carrier. Recipient endothelium was also hydrodissected from the limbus using trypan blue to stain the Descemet's membrane. Continuous curvilinear descemetorhexis (CCD) was performed to remove a circular section of the Descemet's membrane using a 27 gauge cystotome. Donor tissue was inserted into the anterior chamber through a 5 mm limbal incision and apposed to the host stroma. Polymers were removed following transplantation. Similar surgical procedures were performed in both rabbits and eyebank eyes. Haematoxylin eosin stains were performed after 28 days in rabbits, and eyebank eyes were fixed immediately following surgery for endothelial cell counts.

Results: Rabbit control eyes demonstrated stromal oedema caused by loss of Descemet's membrane, whereas transplanted eyes had clear corneas. The mean (standard deviation) pachymetry of operated eyes was 376.6 (SD 32.5) μm compared with 389.6 (SD 25.1) μm in the unoperated eye. Mean endothelial density immediately following surgery in eyebank eyes was 2749 (SD 288) cells/mm^2.

Conclusions: Transplantation of Descemet's membrane by CCD produces a functional graft with an optically clear interface similar to control cornea.

Penetrating keratoplasty (PKP) has a long history as a surgical technique to treat irreversible opacification of the cornea. PKP is still the first choice of surgery for a wide range of diseases including bullous keratopathy, dystrophies, keratoconus, and trauma. However, damage spanning the entire thickness of the cornea from epithelium to endothelium is a relatively rare occurrence. For diseases other than bullous keratopathy, techniques such as deep lamellar keratoplasty (DLKP)[1 2] or epithelial sheet transplantation[3 4] are new alternatives to PKP in which only damaged tissue is surgically replaced. Similarly, the stroma and epithelium are not necessary in the treatment of bullous keratopathy, where only the endothelium is compromised by disease or injury. Several studies have shown the feasibility of transplanting posterior lamellar tissue instead of PKP.

One such technique is endothelial lamellar keratoplasty which uses a microkeratome to create a flap, followed by the replacement of posterior lamellar tissue taken from a donor cornea.[5 6] The use of a microkeratome leaves little opacification of the stromal interface, and a large flap covering the donor offers greater adhesive strength than running sutures used in PKP. Another method for transplanting endothelial tissue with a thin layer of stroma used as a carrier was reported by Melles *et al*.[7] The technique was further refined to allow transplantation through a small limbal incision—as opposed to a large wound reported in the original technique.[8] Initial clinical results are promising; however, manual lamellar incision of the stroma may cause mild haze in the stromal interface.

The adhesion between Descemet's membrane (DM) and posterior stroma is not anatomically strong, so the membrane can be peeled off without much effort. This is done when removing donor endothelium from a DLKP donor button before transplantation.[9] DM can also be detached by hydrodissection or viscodissection to obtain a smooth interface at the plane separating DM and stroma.[10] Our experience with DLKP by hydrodissection led to a new technique to obtain controlled sizes of DM by hydrodissecion followed by continuous curvilinear descemetorhexis (CCD). However, Descemet's membrane alone has the tendency to curl into a tube-like structure with the endothelial cells facing outwards. In order to control the plasticity of DM, we used polymers as a carrier during insertion and spreading of DM. In the present study, we show the histopathological and functional results of endothelial transplantation in rabbit and human eye bank eyes by this technique.

METHODS

Japanese white rabbits (female, 3 kg body weight, Shiraishi experimental animal breeding farm, Tokyo, Japan) were used as an animal model for endothelial transplantation. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. Recipient animals (n = 4) were anaesthetised with 4 ml intramuscular ketamine and xylazine (1:7 mixture) and topical xylocain. To limit the extensive fibrin response observed in rabbits, pupils were expanded with topical tropicamide, and the anterior chamber was irrigated with heparin (5 U/ml). A 5 mm incision was made at the corneoscleral rim down to the plane of Schlemm's canal, and DM was hydrodissected by injecting 0.04% trypan blue into the supra Descemet space using a blunt 27 gauge needle (fig 1A–C). Following injection of air into the anterior

Abbreviations: CCD, continuous curvilinear descemetorhexis; DLKP, deep lamellar keratoplasty; DM, Descemet's membrane; HE, haematoxylin eosin; PKP, penetrating keratoplasty.

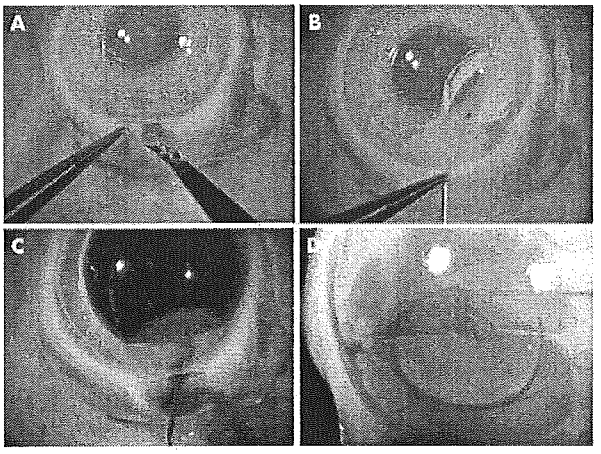

Figure 1 Surgical procedure for preparing the recipient shown in a rabbit eye. (A) An incision was made down to the plane of DM. (B), (C) Hydrodissection of DM is performed with balanced salt solution followed by trypan blue. (D) After removing trypan blue dye, a cystotome is inserted into the anterior chamber to perform CCD. The same procedure was performed in eyebank eyes.

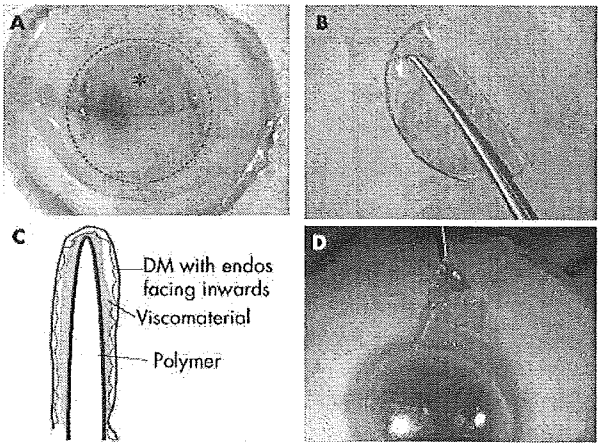

Figure 2 Preparation of donor tissue shown in an eyebank eye. (A) Donor DM is dissected with indocyanine green as described in figure 1, processed into a corneoscleral button, and trephined so that an incision is made in DM (dotted circle). Viscomaterial is coated on the endothelial surface and a hyroxyethyl methacrylate polymer carrier (*) is folded in half and placed on DM. (B) Capsulorrhexis forceps used to grasp the polymer/DM composite with endothelial cells facing inwards. (C) A cross sectional schematic view of polymer/DM shown in (B). (D) The polymer is removed after DM is placed in position with the use of air. The same procedure was performed in rabbits.

chamber, CCD was performed to remove a circular section of the Descemet's membrane using a 27 gauge cystotome (fig 1D).

Two animals were sacrificed using an overdose of pentobarbital at the time of surgery to provide a total of four donor buttons. Following enucleation of the donor globe, a 3 mm incision was made at the corneoscleral rim, and DM was hydrodissected by injecting 6.25 mg/ml indocyanine green so that the donor and recipient DM could be differentiated by colour. After creating a corneal scleral button, a 6 mm diameter incision was made in the DM using a manual trephine, and the endothelium was coated with viscoelastic material to protect the cells from physical friction. The polymer/DM composite was folded in half using an 8 mm diameter hyroxyethyl methacrylate polymer (one day ACUVUE; Johnson and Johnson, Jacksonville, FL, USA) as a carrier (fig 2A). The polymer/DM with endothelial cells facing inward was grasped with capsulorrhexis forceps towards the leading edge of the polymer (fig 2B, C). Donor tissue with the polymer carrier was inserted into the anterior chamber filled with air through the 5 mm limbal incision, and apposed to the host stroma by expanding the polymer. Additional air is injected into the anterior chamber to further adhere the DM to the posterior stromal surface. Polymers were removed at the end of surgery. Control animals ($n = 4$) were operated by CCD alone without transplantation of donor endothelium, and another four rabbits served as sham negative control. All animals received topical antibiotics (levofloxacin) and steroids (betamethasone). After observing the rabbits for 28 days, animals were sacrificed and recipient corneas were fixed with 10% formalin over night. Paraffin sections were stained with haematoxylin and eosin.

Human eye bank eyes (five donors globes, five recipient globes) were obtained from the Northwest Lions Eyebank (Seattle, WA, USA). Donor DM were transplanted into five globes according to the procedure described above. Transplanted eyes were immediately processed into corneal scleral buttons for histological analysis of the transplanted endothelium. Following fixation with 4% paraformaldehyde, endothelial cell density was calculated by staining the nuclei of cells with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, Dojindo Laboratories, Kumamoto, Japan) and analysed using the NIH Image software (National Institute of Health, Bethesda, MA, USA).

RESULTS

Endothelial transplantation in rabbits

The left eyes of four rabbits were transplanted with donor DM. Figure 3A shows a transplanted eye following surgery; the transplanted DM is stained green and the host DM is stained blue. Figure 3B shows the transplanted eye with a clear central cornea 28 days following surgery. Control eyes (fig 3C) had extensive stromal oedema with minimal view of the anterior chamber. The mean (standard deviation) pachymetry reading of transplanted eyes was 407.2 (SD 63.0) μm, which was not significantly different compared with sham operated controls with an average thickness of 391.2 (SD 20.8) μm. Positive control eyes operated by CCD alone were too oedematous to obtain readings with the pachymeter used in our study. The mean cell density in the rabbit eye 28 days after surgery was 2201.3 (SD 441.5) cells/

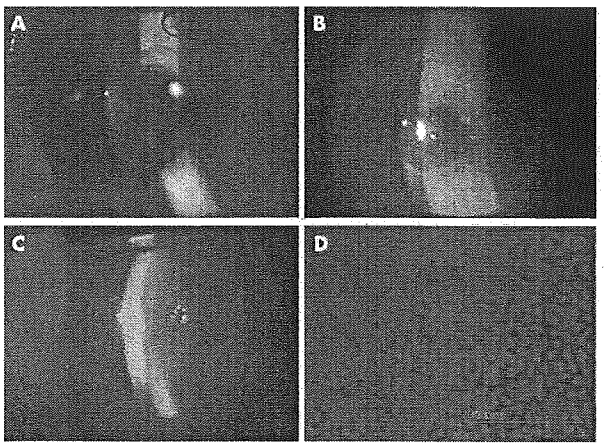

Figure 3 Postoperative eye in a rabbit with DM transplantation immediately following surgery (A) and after 28 days (B). Control eyes stripped of DM had extensive stromal oedema and opacification (C). Histology of endothelium within the graft shows a uniform layer of endothelium (D).

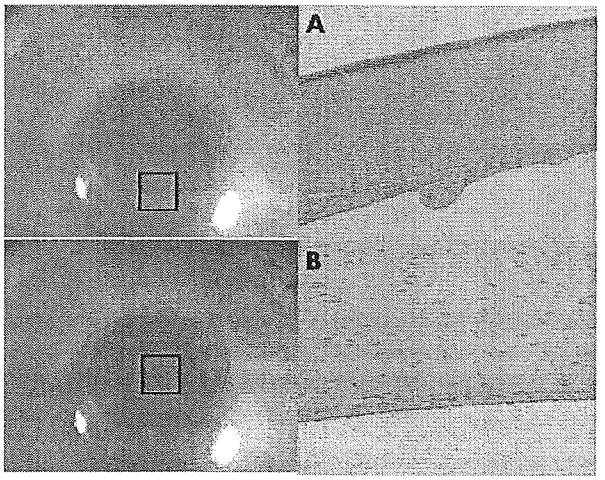

Figure 4 HE stains of the edge (A) and centre (B) of donor show a well apposed DM with slight curling of the edge. Original magnification (×100) for HE stains.

mm^2 (fig 3D) compared with 3180.5 (SD 98.2) cells/mm^2 in sham control. Cell loss was greater in rabbit eyes compared with eye bank eyes because of the very shallow anterior chambers in rabbits, which was an obstacle during surgical procedures.

Animals were sacrificed after 28 days, and the transplanted eyes were processed for histological examination. Haematoxylin eosin (HE) stains of the peripheral graft show a slightly curled edge of the transplanted DM (fig. 4A). The central graft is attached to the host stroma (fig 4B), which under high magnifications shows how the transplanted DM resembles DM in sham operated controls (fig 5). There was no observable infiltration of inflammatory cells in any of the operated eyes after 28 days.

Endothelial transplantation in human eyebank eyes

A total of five "donors" were transplanted into another five eyebank eyes using the same procedure explained in detail above. Corneal scleral rims were processed immediately following implantation to show the positioning of the graft (fig 6). Nuclear stains using DAPI revealed an endothelial cell density of 2749 (SD 288) cells/mm^2, suggesting that an adequate density of endothelial cells can be transplanted using this technique. No information was available as to the cell density of the eyes before the experiment; therefore the endothelial cell loss rate was not calculated.

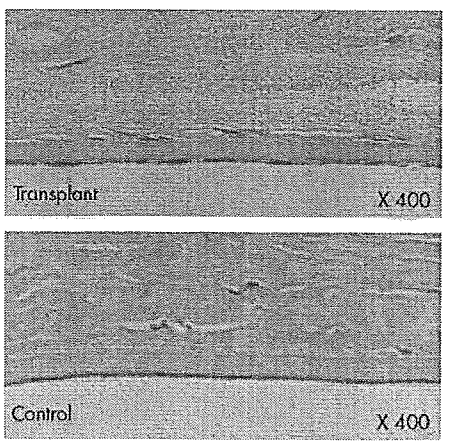

Figure 5 Higher magnification (×400) of the central graft reveals similar histological findings in both the transplanted eye and control.

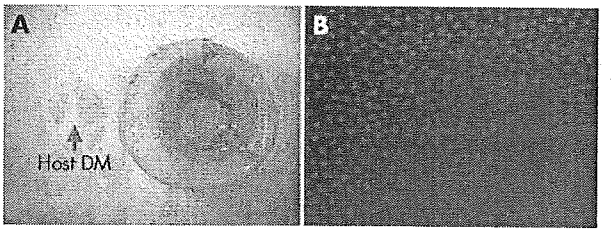

Figure 6 DM transplantation in human eyebank eye with green donor DM and blue recipient DM following CCD (A). Average endothelial density immediately following insertion of donor DM was 2749 (SD 288) cells/mm^2.

DISCUSSION

Surgical techniques in keratoplasty have changed over the past few years towards reducing tissue damage to a minimum, while transplanting only the layers of the cornea that are necessary for the particular patient. For stromal diseases such as keratoconus and the various dystrophies, DLKP can remove all of the stroma while leaving the host endothelium intact.[1,2] Epithelial transplants using amniotic membrane carriers are already in clinical use for the treatment of acute phase burns and Stevens Johnson syndrome with persistent epithelial defects.[11] The same technique can also be used to restore vision in the chronic stage of cicatricial disease such as ocular cicatricial pemphigoid, if the indications are chosen carefully.

Bullous keratopathy, however, is still mainly treated by PKP as the techniques involved in transplanting endothelium were believed to be technically difficult. Several experimental reports using animals have attempted to transplant endothelium by seeding the cells on hydrogel polymers,[12] or by using bovine DM as carriers.[13] More recently, clinical studies have described techniques to transplant the posterior stroma, including the endothelium, into bullous keratopathy patients. One technique is reported by Azar *et al*, who used a microkeratome to create a flap so that the posterior half of the donor cornea could be transplanted beneath the flap.[6] Our experience with the technique is favourable, with a clear optical interface between the host and donor. However, epithelial ingrowth can be a complication, and the need of a keratome can be costly. Another method described by Melles *et al* uses a spatula to dissect the host Descemet's membrane, after which a donor DM is inserted into the anterior chamber though a small corneal scleral incision.[7,8] The procedure seems promising in terms of refraction; however, stromal haze in the donor-host interface may occur when a spatula or knife is used to create a lamellar incision in the deep stroma. A custom made scraper was recently reported by the same group, which may solve these issues in the repicient stromal bed.[14]

The method described in this study uses hydrodissection to dissociate Descemet's membrane in both the donor and host, without the use of special instruments. This creates clear cut dissociation at the interface of DM and stroma, because fluid enters into the plane of weakest adhesion. The interface in this case is smoother than other methods using blades or spatula and, furthermore, does not interrupt the posterior stroma that contains keratocytes which may become activated to cause tissue reaction. The high magnification in figure 5 shows how the transplanted DM is closely apposed to the host stroma with little cellular infiltration or scarring in the posterior stroma. The grafts in rabbits show no interface

opacity under slit lamp examination, and the endothelial cell count is within an acceptable range. The average cell density in rabbits was lower than human eyebank eyes in our study, which was due to the fact that surgical manipulation was much more difficult in rabbits, which have very shallow anterior chambers and show fibrinotic response during surgery.

The rabbit data show that transplanted DM is functional, and that little tissue reaction is observed at the host-donor junction. However, as rabbit endothelial cells are known to proliferate in vivo, the same experiment was done using human eyebank eyes. The objective was to simulate a clinical situation, and also to assess the loss of endothelial cells during the procedure. As shown in figure 6, controlling the size and apposition of donor DM is possible, and the average endothelial cell density was above 2500 cells/mm^2. The preoperative endothelial density was not available; however, considering that the eyes were preserved in moist chambers without the use of storage medium, the results can be said to be more than adequate.

The largest obstacle to manipulating DM is the tendency for the 10 μm thick membrane to curl up with the endothelium facing outwards. Melles *et al* have developed an injector for the purpose of inserting the membrane into the anterior chamber.[10] We have shown that a hydroxyethyl methacrylate polymer can be used as a carrier by folding the DM in half, and also protect the graft from the loss of endothelial cells during insertion into the anterior chamber. The polymer can then be unfolded in the anterior chamber, and also be used to appose the DM to the stroma. The inserted DM is unfolded using a spatula inserted into the plane opposite the endothelium. Although the donor edge is not necessarily a perfect fit with the recipient bed (fig 4), the adhesion of donor DM is firm as evidenced from the fact that the membrane remains attached following paraffin fixation during HE stains. The report by Melles *et al* also shows that a perfect match is not necessary.

Transplantation of hydrodissected DM is an effective means to reproduce the normal anatomy of the posterior stroma. The hydrostatic pressure exerted by the endothelial pump is sufficient for the immediate attachment of the donor DM, and a polymer carrier offers protection and a means to insert DM through a small surgical wound. Encouraged by the results of this study, we are currently preparing a clinical trial based on the surgical technique described here.

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Deep Lamellar Keratoplasty (DLKP) in Keratoconus Patients Using Viscoadaptive Viscoelastics

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Purpose: To demonstrate the effectiveness and safety of using viscoadaptive viscoelastics in deep lamellar keratoplasty (DLKP) for keratoconus.

Methods: A nonrandomized, comparative, interventional case series was performed on 12 eyes of 10 consecutive keratoconus patients without a history of acute hydrops. Patients were treated with DLKP using ophthalmic viscosurgical devices (OVDs) to dissociate Descemet membrane (DM) from the stroma before suturing of the donor cornea. Eight eyes were operated using Viscoat® (dispersive), and 4 eyes were operated using Healon V® (viscoadaptive).

Results: Six out of 8 eyes in the Viscoat group were successfully treated, and 2 eyes were converted to PKP because of tears in the DM. Another 3 eyes had double chambers as a result of perforations or tears in DM, which were treated by injecting air into the anterior chamber. All 4 eyes in the Healon V group were successfully operated, with 1 case with microperforation of DM during suturing. Healon V offered better control of the area to be dissociated and served as a pseudostroma that protects the exposed DM while the overlying stroma is maneuvered.

Conclusion: Healon V is a valuable tool for performing DLKP in difficult cases such as keratoconus.

Key Words: Healon V, Viscoat, deep lamellar keratoplasty

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Deep lamellar keratoplasty (DLKP) is a technique that preserves the endothelium in patients suffering from corneal stromal disease. The replacement of endothelium by penetrating keratoplasty (PKP) in such a case not only is unnecessary but places the patient at risk for endothelial rejection. This is especially true for keratoconus patients, who are generally young and socially active. Although the 5-year success rate of PKP in keratoconus is over 95% in most studies, episodes of endothelial rejection can occur in approximately 20%.[1–3] Even without a history of immunologic rejection, endothelial density slowly declines over a period of years, which may require further keratoplasty during the lifespan of the patient.

DLKP offers better optical clarity than lamellar keratoplasty (LKP) while avoiding the risk of endothelial rejection associated with PKP. Although DLKP is theoretically ideal for young keratoconus patients, many surgeons still do not choose DLKP because of its technical difficulty and long surgery time. DLKP can especially be time consuming when stromal tissue is removed layer by layer until the Descemet membrane (DM) is exposed.[4–7] However, Melles et al introduced a revolutionary technique to directly visualize DM using the interface of an air bubble injected into the anterior chamber.[8] They further refined the technique by devising new instruments and using viscoelastics to dissociate DM.[9] This and other modifications of the technique have allowed DLKP to be performed safely, even for therapeutic cases with perforated corneas.[10,11]

Keratoconus patients, however, are still the most difficult cases to treat with DLKP because of the relatively higher risk of rupturing DM during surgery.[12] Although the problem can be managed by air tamponades in most cases, the complication should be avoided because it may cause damage to the endothelium.[13] From our experience with DLKP, microperforation of DM has occurred during suturing. Larger tears of DM were observed during viscodissection, either from excessive amounts of viscomaterial or from spreading of a side port incision. Inadvertent perforation while removing stromal tissue is also a characteristic of DLKP in keratoconus. In this study, we show how the characteristics of a new viscoadaptive ophthalmic surgical device, Healon V, can be used to avoid many of the problems associated with DLKP in keratoconus patients.

METHODS

Patients

Twelve eyes of 10 consecutive patients with moderate to severe keratoconus were enrolled in the study. Patients consisted of 6 men and 4 women with an average age of 30.5 (range 20 to 47). Patients with a history of acute hydrops were excluded because DM is often lacerated and scarred from wound healing. All surgeries were performed by one surgeon (S.S.) between April 2002 and July 2003 at either Ichikawa General Hospital (Chiba, Japan) or Ryogoku Eye Clinic (Tokyo, Japan) using a similar surgical setup. Thorough ocular exams were preformed before and following surgery, and specular micrographs were recorded when possible. All surgeries were performed using the same technique described below, except for the fact that 2 different ophthalmic viscosurgical

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