Conjunctival Transdifferentiation Is due to the Incomplete Removal of Limbal Basal Epithelium

Friedrich E. Kruse, James J. Y. Chen, Ray J. F. Tsai, and Scheffer C. G. Tseng

Previous studies have shown that using n-heptanol to create a total corneal epithelial defect beyond the limbus results in two different healing patterns with an unpredictable incidence. Between 14–68% of these wounded rabbit corneas (n = 287, combining various reports) showed extensive vascularization and conjunctivalization, whereas the remaining were not vascularized and had conjunctival transdifferentiation with a cornea-like epithelium. To investigate the role of the limbal epithelium in these two healing patterns, the authors treated rabbit eyes for various durations with n-heptanol and additional scraping. Histology showed that treatment for up to 120 seconds removed both the corneal and conjunctival epithelia but left the limbal basal cells intact. To prove viability, they cultured the treated limbal explants on collagen gel. After 14 days of culture, increased stratification of the limbal epithelium and an epithelial outgrowth onto the corneal stroma was observed. The latter was proven to be of corneal origin (positive to AE-5 but negative to AM-3 monoclonal antibody staining). The authors then surgically removed the entire limbal zone including 2 mm of peripheral cornea and 3 mm of adjacent conjunctiva in addition to n-heptanol debridement of the entire corneal epithelium in 54 rabbit eyes and observed a high incidence (96%) of corneal vascularization and conjunctivalization of the resultant epithelial phenotype (positive to AM-3, but negative to AE-5 monoclonal antibody staining). These results support the hypothesis that corneal epithelial stem cells are located in the limbus and indicate that an incomplete removal of the basal limbal epithelium by n-heptanol leads to unvascularized corneas with conjunctival transdifferentiation. Conversely, complete removal of such cells results in corneal vascularization and conjunctivalization. Invest Ophthalmol Vis Sci 31:1903–1913, 1990

Large corneal epithelial wounds involving the limbus have been studied in various rabbit models.1–4 These large defects can be created either by mechanical1 or chemical (n-heptanol) debridement.2–4 When the limbal epithelium is removed, the general assumption is that the denuded corneal surface is healed by the ingrowth of the surrounding conjunctival epithelium.1–4 Previous studies show that some of the corneas healed with extensive vascularization, and others healed either without vascularization or with vascularization limited to the peripheral cornea.1–4 In the absence of corneal vascularization, the healing epithelium first shows a conjunctiva-like morphology containing goblet cells, which later transforms into a cornea-like epithelial morphology without goblet cells.1–6 This process is called "conjunctival transdifferentiation." However, in the presence of corneal vascularization, the epithelium retains a conjunctiva-like morphology without conjunctival transdifferentiation, and the resultant epithelium remains conjunctivalized1,4,7,8 even several months after wounding.4

The mechanism of conjunctival transdifferentiation has been studied by numerous investigators.1–11 Except for the earlier work by Friedenwald,1 all other investigators including ourselves2–11 used n-heptanol debridement to create a total corneal epithelial wound in rabbits. Interestingly, this technique induced an unpredictable and variable occurrence of conjunctival transdifferentiation, corneal vascularization, and conjunctivalization. The incidence of the latter varied in the previous reports from 14%, when n-heptanol was applied for 30 seconds,5 to 68%, when the agent was applied for 60 seconds together with mechanical scraping with a surgical blade.11 As a possible explanation for this variability, we previously suggested that it could be caused by a variable degree of tissue damage.4 Nevertheless, the extent of the removal of limbal epithelium by n-heptanol, especially the limbal basal cells, has never been investigated.
Incomplete removal of the limbal basal cells seems to be of particular importance because some of these cells are believed to be the stem cells of the corneal epithelium.\textsuperscript{12,13} The concept and application of limbal stem cells has recently been reviewed.\textsuperscript{14} Stem cells represent the proliferative source for all differentiated cells and are responsible for the ultimate replacement and regeneration of such a highly self-renewing tissue as the corneal epithelium.\textsuperscript{15,16} Based on this notion, it is crucial to know whether or not the limbal basal cells were completely removed in chemical debridement with n-heptanol. One can furthermore speculate that a complete removal of the limbal basal cells will lead to vascularization and conjunctivalization of corneas, whereas an incomplete removal of these cells can induce the process of conjunctival transdifferentiation.

To examine this hypothesis, we specifically investigated n-heptanol's ability to remove the entire limbal basal epithelium. A tissue culture of the treated limbal explant was used to investigate the viability of the remaining cells and their differentiative and proliferative capacity. Furthermore, we created a rabbit model with total removal of limbal epithelium by surgical lamellar dissection and examined the incidence of corneas that undergo vascularization and conjunctivalization.

**Materials and Methods**

All investigations were done according to the ARVO Resolution on the Use of Animals in Research.

**Materials**

Ham's F12 medium, fetal calf serum, and Dulbecco's modified essential medium (DMEM) were obtained from GIBCO (Grand Island, NY). Mouse epidermal growth factor (mEGF), bovine insulin, dimethylsulfoxide (DMSO), cholera toxin, and the secondary antibody (goat anti-mouse IgG fluorescein conjugated) were purchased from Sigma (St. Louis, MO). Collagen solution was prepared from rat tails using a method similar to that of Elsdal and Bard\textsuperscript{17} as described in our previous report.\textsuperscript{18} Culture dishes were purchased from Becton and Dickinson (Falcon 3046; Oxnard, CA).

**Methods: Conventional model by n-heptanol removal**

New Zealand albino rabbits of either sex, weighing 2–3 kg, were anesthetized by intramuscular injection of xylazine hydrochloride (50 mg) and ketamine hydrochloride (50 mg). The eye was proptosed, and the entire corneal epithelium and 3 mm of the adjacent limbal and conjunctival epithelium were removed in a manner similar to the technique described by Cintron et al.\textsuperscript{18} A cotton-tip applicator was dipped into n-heptanol solution until it became completely wet and was used to rub the corneal surface thoroughly including the limbus and 3 mm of the adjacent conjunctiva. The rubbing was done in a circular fashion for durations of 30, 60, 90, 120, 180, and 300 sec. After this procedure, the eye was washed with sterile phosphate-buffered saline (PBS) to remove excess n-heptanol. The cornea, limbus, and adjacent conjunctiva were then scraped several times with a Bard-Parker surgical blade to achieve complete removal of the epithelial tissue. Three rabbit eyes were studied for each duration. After treatment, the animals were killed by an intravenous overdose of pentobarbital. The anterior segment of the eyes, including the iris and lens, was transferred to a sterile culture dish with PBS. The iris and lens were then removed, and the central cornea was excised using a 6-mm trephine. The remaining corneoscleral ring was then dissected into 12 equal pieces in a clockwise fashion. Every other piece was processed for frozen sections, and the remaining six pieces were used for explant culture.

**Explant Culture for Epithelial Outgrowth**

To test the viability of the remaining limbal basal epithelium, the remaining six pieces of the eyes treated for 60 or 120 sec were then placed endothelial-side down on culture dishes which had previously been coated with collagen gel.

The preparation and gelling of the collagen gel has been described in a previous report.\textsuperscript{18,19} Briefly, an ice-cold stock solution of collagen prepared from rat tails was mixed with 5 X concentrated DMEM medium and 0.34 M NaOH in a volume ratio of 8:2:1 at 4°C and suspended onto the culture dishes immediately before the experiment. A complete gel was achieved by incubating the mixture at 37°C for 1 hr. The cultures were covered with Ham's F12 and DMEM medium (1:1), containing 2 ng/ml of mEGF, 1 \( \mu \)g/ml of insulin, 0.1 \( \mu \)g/ml of cholera toxin, 10% fetal calf serum, 0.5% DMSO, 50 \( \mu \)g/ml of gentamicin, and 1.3 \( \mu \)g/ml of amphotericin B. The culture dishes were incubated at 37°C under 5% CO\textsubscript{2} and 95% air with 94% humidity. Culture media were changed every second day, and the cultures were observed daily under an inverted phase-contrast Nikon Diaphot microscope (Nikon, Garden City, NY). When epithelial outgrowth onto the collagen gel was observed, the explant, including the underlying colla-
gen gel, was excised with a razor blade and processed for frozen sections.

New Model by Surgical Removal

To achieve a complete removal of limbal basal epithelial cells, the limbal tissue was removed in a ring fashion from one eye of a total of 54 rabbits. In detail, the excision was done first on the peripheral cornea, 2 mm within the limbus, and followed by a superficial lamellar dissection toward the limbus. A 360° conjunctival peritomy was then performed 3 mm beyond the limbus. The total excision of limbal tissue was completed with scissors. By doing so, we were certain that the depth of the excision was below the basement membrane, extending into the corneal stroma and the substantia propria, to ensure the total removal of all limbal basal cells. After the surgical excision of the limbal tissue, corneal epithelium was debrided using n-heptanol as described. After surgery, the eyes were treated with topical gentamicin, three or four times a day for week 1 and two times a day for week 2. The injured eyes were examined weekly using hand-held light examination and external photography until completely healed, and then monthly up to 10 months. At various times, randomly selected animals were killed by an intravenous overdose of pentobarbital, and the corneoscleral button of the injured eye was processed for frozen sections to evaluate the resultant epithelial phenotype.

Morphologic and Immunohistochemical Studies

Frozen sections from both the surgically and n-heptanol-treated rabbit eyes and from the explant culture were stained with hematoxylin and eosin, and primary monoclonal antibodies were applied. Monoclonal antibody AE-1 reacts with 56-kilodalton (kD) and 50-kD keratins which are prevalent in basal epidermal keratinocytes. As shown in a previous report, this antibody also stains rabbit conjunctival epithelial cells and human corneal epithelial cells. The monoclonal antibody AE-5 recognizes a major corneal 64-kD keratin expressed by all cell layers of the rabbit corneal epithelium and by the suprabasal layers of the limbal epithelium. Both of these monoclonal antibodies were a gift from T-T Sun. The monoclonal antibodies, APSM-1, APSM-2, and AM-3, have recently been developed in our laboratory. Both APSM-1 and APSM-2 were developed accidentally in the pursuit of monoclonal antibodies against ocular mucin using the immunization of porcine stomach mucin (PSM). Female BALB/c mice were inoculated subcutaneously with PSM (Sigma) 1 mg of protein/ml in PBS, emulsified with an equal volume of Freund's adjuvant (Difco, Detroit, MI). The mice were boosted with the same solution 1 and 2 months after the initial immunization. Three to 5 days after an intraperitoneal injection of mucin/PBS, a fusion of splenocytes with P3-N51-Ag4-1 (NS-1) myeloma cells was done using 1 ml of 50% polyethylene glycol at 37°C for 90 sec. Resultant hybridomas were selected by sequential growth in DMEM plus 15% FBS supplemented with hypoxanthine/aminopterin/thymidine, or hypoxanthine/thymidine. Supernatants from the hybridomas were screened using frozen sections of normal rabbit cornea–limbus–conjunctival strips. As shown in Figure 1A, APSM-1

FIG. 1. Immunofluorescence staining of the normal conjunctival, limbal and corneal epithelia with APSM-1 (A) and APSM-2 (B). The limbus is indicated by an arrow of which the arrowhead is placed underneath the corneolimbal junction and the arrow points to the corneal direction.
Fig. 2. Frozen section of normal rabbit conjunctiva, limbus and cornea (A) and those after n-heptanol treatment followed by scraping for 30 sec (B), 60 sec (C), 90 sec (D), 120 sec (E), and 180 sec (F). A higher magnification of the above sections at the limbal zone are shown in (G), (H), (I), (J), (K), and (L) respectively. The limbus is indicated by a bar in (A–F). (Hematoxylin-Eosin).
stains the limbal basal and the nongoblet basal conjunctival epithelium. Figure 1B shows that APSM-2 stains the suprabasal cell layers of the limbal epithelium and most of the conjunctival nongoblet epithelial cells and some goblet cells. The monoclonal antibody AM-3 recognizes the nonglycosylated portion of the corneal protein of ocular mucin and therefore stains the conjunctival goblet cells specifically. All three antibodies, APSM-1, APSM-2, and AM-3, do not recognize normal corneal epithelium. A monoclonal antibody to vimentin (Chemicon International, Los Angeles, CA) was used to identify mesenchymal cells. After application of the undiluted supernatant of these antibodies, a 1:200 dilution of the secondary antibody fluorescein-conjugated goat antimouse IgG was added. The slides were examined and photographed using a Zeiss Axiophot fluorescence microscope (Oberkochen, West Germany) and Kodak TMX 400 film (Rochester, NY).

Results

Extent of Removal of Limbal Epithelium after n-Heptanol Treatment In Vivo

After n-heptanol treatment, frozen sections from the 2, 4, 6, 8, 10, and 12 o'clock position were analyzed histologically. All sections for each duration showed a similar removal of the epithelium of the corneal, limbal, and conjunctival zones. Therefore, representative sections for durations of 30, 60, 90, and 120 sec are shown in Figure 2. The amount of the residual epithelial cells in these three zones is summarized in Table 1.

Compared with the normal control (Figs. 2A, G), n-heptanol treatment for 30 sec removed some superficial corneal, limbal, and conjunctival epithelial cells, but it left intact the corneal basal cell layer and 3-5 cell layers of limbal epithelium (Figs. 2B, H). This pattern was observed in all 18 (100%) specimens. n-Heptanol treatment for 60 sec led to the removal of almost the entire corneal epithelium in all 18 (100%) specimens, with some scattered basal corneal cells left (Fig. 2C). However, the basal 3-5 cell layers of the limbal epithelium remained intact (Fig. 2I). When the treatment was extended to 90 sec, most of the corneal and conjunctival epithelial cells were removed (Fig. 2D). In the limbal zone, 2-3 cell layers still remained intact (Fig. 2J) in 15 of 18 (83%) of the specimens. When the treatment was applied for 120 sec, corneal epithelial cells were removed totally, but the limbal basal epithelial cells remained adherent (Figs. 2D, K) in 11 of 18 (60%) of the specimens. When the treatment was continued for 180 sec, the corneal epithelium was removed completely in all 18 specimens. One or two interrupted cell layers of limbal epithelium were observed in only 3 of 18 (17%) of the specimens, and no limbal epithelium was observed in the remainder (Figs. 2F, L). After 300 sec of treatment, both the corneal and limbal epithelia were entirely removed.

Outgrowth of Epithelial Cells in Explant Culture

To prove that the remaining limbal basal epithelial cells were able to proliferate and differentiate, specimens from every second clock-hour zone were cultured for 14 or 28 days. After 60 or 120 sec of treatment, occasional cellular outgrowth from the explant onto the collagen gel was observed on day 2 of culture. These cells seemed to be fibroblastic, based on their morphologic appearance. A substantial outgrowth of these cells was observed on day 3 of culture, followed by an outgrowth of smaller and rounder cells of probably epithelial or endothelial origin. This outgrowth pattern was observed predominantly on the collagen gel adjacent to the limbal and corneal part of the specimen. After 4 weeks of culture, the outgrowing cells had migrated further to the periph-

Table 1. Summary of the residual epithelial layer of corneal, limbal, and conjunctival zone following n-heptanol treatment

<table>
<thead>
<tr>
<th>Duration of n-heptanol treatment (sec)</th>
<th>Conjunctival epithelium</th>
<th>Limbal epithelium</th>
<th>Corneal epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2-3 cell layers</td>
<td>5-8 cell layers</td>
<td>4-6 cell layers</td>
</tr>
<tr>
<td>30</td>
<td>1 or 2 cell layers</td>
<td>3-5 cell layers</td>
<td>Predominant basal cell layer</td>
</tr>
<tr>
<td>60</td>
<td>Predominant basal cell layer</td>
<td>2-3 cell layers (83%)</td>
<td>Scattered basal cells</td>
</tr>
<tr>
<td>90</td>
<td>Scattered single basal cells</td>
<td>2-3 cell layers (60%), No cells (39%)</td>
<td>No cells</td>
</tr>
<tr>
<td>120</td>
<td>Scattered single basal cells</td>
<td>2 cell layers (17%), No cells (83%)</td>
<td>No cells</td>
</tr>
<tr>
<td>180</td>
<td>Scattered single basal cells</td>
<td>No cells</td>
<td>No cells</td>
</tr>
<tr>
<td>300</td>
<td>No cells</td>
<td>No cells</td>
<td>No cells</td>
</tr>
</tbody>
</table>

No percentage is given if the observation was uniform in all 18 specimens, otherwise a percentage is provided in parenthesis.
ery, and the proportion of fibroblastic cells had increased. The outgrowth pattern of all cultures from the specimens treated for 60 sec was similar to that for 120 sec.

To investigate the cellular origin of the outgrowth from both 14- and 28-day cultures, the frozen sections of the outgrowth were stained with a battery of monoclonal antibodies. Figure 3 summarizes the immunofluorescent staining pattern of an explant that was treated with n-heptanol for 120 sec and cultured for 14 days. The multilayer epithelial outgrowth on the surface of the denuded corneal stroma and the entire (full-thickness) limbus (Fig. 3A) was strongly positive for AE-5, the monoclonal antibody marker for corneal epithelium (Fig. 3B). This result indicates that the outgrowth was of corneal epithelial phenotype. AE-1, APSM-1, and APSM-2 are monoclonal antibodies that strongly recognize normal limbal epithelium. The positive staining to these three antibodies in the limbal region (indicated by a bar in Figs. 3C-E for AE-1, APSM-1, and APSM-2, respectively) and relatively weak staining in the outgrowth over the denuded stroma support the fact that the remaining limbal basal epithelium was still viable. The mucin-specific monoclonal antibody, AM-3, did not show any staining on the epithelium at the limbal region or on the outgrowth, indicating that the epithelial outgrowth was not a conjunctival derivative (Fig. 3F). The outgrowth on the collagen gel was negative for these antibodies, indicating that the outgrowing cells were not of corneal or conjunctival origin. On the contrary, these outgrowing cells could be stained with a vimentin antibody, suggesting their endothelial or stromal derivation (data not shown).

Healing of a Total Epithelial Defect after Surgical Removal of the Limbus

To determine if complete removal of limbal basal epithelium results in the absence of conjunctival transdifferentiation, the perilimbal corneal and conjunctival epithelia were surgically removed from 54 eyes. The animals were followed up to 10 months. Fifty-two of 54 eyes (96%) had abnormal wound healing that could be characterized by delayed healing, recurrent erosions, and corneal vascularization. Figure 4 illustrates such an abnormal healing pattern from one representative rabbit eye. The defect persisted more than 1 month with minimal healing. Corneal vascularization occurred shortly after injury and became intense one month later (Figs. 4A, B versus C, D). The vascularization progressed with increasing intensity (Figs. 4E, F versus G, H). During the entire follow-up period, we observed recurrent erosions by fluorescein staining (Figs. 4F, H). Due to recurrent erosions and chronic inflammation, a granuloma pyogenicum usually developed (indicated by * in Fig. 4G).

The resultant epithelial phenotype in these 52 rabbit eyes was proven to be conjunctival, not only by
Fig. 4. External appearance of one representative rabbit cornea after surgical removal of the entire limbus and n-heptanol debridement of the corneal epithelium. Corresponding fluorescein stainings are shown in the right column. After 2 weeks of treatment some new vessels are found (A) as well as delayed wound healing of the epithelial defect (B). After 1 month, increasing vascularization (C) and recurrent epithelial defect (D). Two and one-half months after wounding a different pattern of vascularization (E) and a recurrent erosion (F) was noticed. After 5 months, a granuloma pyogenicum was found (G) (*) along with recurrent erosion (H).

histologic studies (data not shown), but also by a positive staining to AM-3, a monoclonal antibody directed against mucin of conjunctival goblet cells (Figs. 5A versus C), and negative for AE-5 staining (Figs. 6A versus C). Two of the 54 rabbits (4%) showed minimal vascularization, and the resultant epithelial phenotype was corneal since it was positive to AE-5 but not AM-3 monoclonal antibody (Figs. 5B, 6B).

Discussion

In self-renewing tissues, stem cells are responsible for ultimate cellular replacement and tissue regenera-
Fig. 5. Immunofluorescence staining of conjunctiva, limbus (as indicated by the arrow) and cornea with AM-3. Normal (A) and transdifferentiated corneal epithelium (B) are negative but conjunctival goblet cells stain positive. The vascularized cornea (C) shows positive staining, indicating the presence of goblet cells in the vascularized corneal epithelium.

Based on studies of hemopoietic cells and keratinocytes, the stem cells are thought to be quiescent in the steady state and have an unlimited proliferative capacity. With tissue demand, they can, after mitosis, give rise to transient amplifying cells (TAC), with a high proliferative capacity but limited life span. Eventually, the TAC cells are differentiated into postmitotic cells that become terminally differentiated.

We once thought that the proliferative source of the corneal epithelium was located in the basal layer and that the peripheral corneal epithelium was important for the renewal of corneal epithelial cells. The phenomenon of centripetal epithelial cell migration in the cornea has been observed after wounding, corneal grafting, and with normal desquamation. This led Thoft et al. to propose the hypothesis that the epithelial cell mass is maintained from the periphery. More recent studies show that the peripheral corneal epithelium has a higher proliferative capacity than the central area. The first evidence to indicate the limbal location of corneal epithelial stem cells was provided by Schermer et al. Lately, additional evidence was provided by Cotsarelis et al. based on the study using prolonged thymidine labeling under stimulation with a tumor promoter.

The new concept of the limbal location for the corneal epithelial stem cells helps clarify the two confusing and unexplained observations of the previous studies in which n-heptanol was used to debride corneal and limbal epithelia. First, two different healing patterns occurred, related to the presence or absence of corneal vascularization. Second, the incidence of these two patterns was unpredictable. We noted that the n-heptanol debridement led to variable removal of the limbal epithelium. As a result, complete removal could explain the occurrence of corneal vascularization and conjunctivalization, and incomplete removal would lead to unvascularized corneas and conjunctival transdifferentiation.

N-heptanol treatment for 60 and 120 seconds removed the entire corneal epithelium, which was consistent with the original finding of Cintron et al. Nevertheless, this treatment was not able to remove all the limbal basal cells even when applied for 120 seconds, longer than the original report of 60 seconds. A complete removal could only be achieved when the treatment was extended for 180 seconds in some eyes and for 300 seconds in all eyes (Table 1). The re-
Fig. 6. Immunofluorescence staining of conjunctiva, limbus (as indicated by the arrow) and cornea with AE-5. Normal (A) and transdifferentiated corneal epithelium (B) stain positive as does superficial limbal epithelium. The vascularized cornea (C) shows only scattered stain; the limbus is negative.

remaining limbal cells retained their differentiative and, to a certain extent, proliferative capacity in an explant culture model. When the entire limbal zone was surgically removed, we noted an overwhelmingly high incidence—96% (52 of 54)—of vascularized corneas with a conjunctival epithelial phenotype. Surgical removal of the limbal zone also damaged the limbal basement membrane or vasculature, which might be necessary for wound healing resulting in a unvascularized cornea with normal corneal epithelium. Therefore, we could not exclude the possibility that conjunctival transdifferentiation might occur in the migrating conjunctival epithelium under the most ideal situation where the removal of limbal basal epithelium had not altered the subjacent microenvironment. Our studies seem to substantiate the important role of the limbal epithelium in the wound healing of large corneal epithelial defects and clarify the two unsolved questions in the previous reports.

Previous reports show that the resultant epithelium after conjunctival transdifferentiation has a corneal-like morphology.\textsuperscript{1,3,4} The transdifferentiated epithelium differs from the normal corneal epithelium in glycogen metabolism,\textsuperscript{32} tensile strength,\textsuperscript{3} keratin-like proteins,\textsuperscript{6} general protein profiles,\textsuperscript{33} and paracellular permeability,\textsuperscript{34} suggesting that the transdifferentiated epithelial phenotype is not genuinely corneal. Furthermore, we also demonstrated that the phenotype is unstable and can be modulated by the presence of vascularization,\textsuperscript{4} photothrombotic occlusion of preexisting vascularization,\textsuperscript{31} and alteration of supply of vitamin A to the corneas.\textsuperscript{9,10}

That complete removal of limbal stem cells leads to the conjunctivalization and vascularization of the cornea helps to explain the pathogenesis of some clinical disorders. Experimentally, the total removal of the limbus allows an ingrowth of conjunctival epithelium and corneal vascularization when further debridements were made on the central cornea.\textsuperscript{35} Even when two thirds of the limbus was removed, a large epithelial defect can still induce delayed healing, conjunctivalization, and vascularization.\textsuperscript{36} These findings explain why similar clinical conditions occur when pathologic insults involve the limbal zones. These disorders include chemical injuries, Stevens-Johnson syndrome, some contact-lens induced keratopathy, and aniridia. The corneas in these disorders always undergo recurrent erosions, chronic inflammation, and vascularization. The impression cytologic survey of these disorders also confirms the exis-
tence of conjunctival epithelial ingrowth with goblet cells.\textsuperscript{3,33} Therefore, these diseases can be regarded as a deficiency or dysfunction of the limbal stem cells. In chemical injuries, this hypothesis can be further substantiated by Hughes' observation that the final outcome depends on the degree of limbal ischemia and corneal epithelial damage.\textsuperscript{39,40} It can also be supported by a recent rabbit experiment by Tsai and Tseng\textsuperscript{41} that showed that conjunctival transplantation, which includes limbal epithelium, called "limbal transplantation," is more effective in restoring the corneal epithelial phenotype than the transplantation of conjunctival tissue alone. After limbal transplantation, phenotypic restoration is accompanied by decreased corneal vascularization and a better corneal epithelial surface, which was also observed in the limbal transplantation of human patients.\textsuperscript{32}

The fact that the explant showed only limited epithelial outgrowth in 14–28 days of culture indicates that the remaining stem cells might not have received optimal growth support in vitro. This is supported by the positive AE-5 immunofluorescent staining extending to the entire limbal epithelium (Fig. 3B), suggesting that the stem cells have lost their "stemness" under the present culture conditions and become differentiated into cells of corneal derivation that express the 64-kD keratin. Factors responsible for the modulation of limbal corneal stem cells are presently unknown. From studies of the hemopoietic stem cells,\textsuperscript{3,34} one could assume that a complex regulatory system consisting of various cytokines in a limbal microenvironment might be in existence.\textsuperscript{45} Since the limbal region is highly vascularized\textsuperscript{46-47} and under neural influence,\textsuperscript{48-50} it is conceivable that the regulatory cytokines might come from the blood and/or may include some neurotrophic factors. To understand the pathophysiology of limbal stem cell dysfunctions better, it is important to study the regulatory mechanisms by which the differentiation and proliferation of limbal stem cells are modulated.

Key words: conjunctival transdifferentiation, cornea, epithelium, limbus, monoclonal antibody, stem cells, wound healing

References